# MOLECULAR MECHANISMS OF ZINC HOMEOSTASIS IN *BACILLUS SUBTILIS* MEDIATED BY ZUR, A FUR FAMILY MEMBER.

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# MOLECULAR MECHANISMS OF ZINC HOMEOSTASIS IN *BACILLUS SUBTILIS* MEDIATED BY ZUR, A FUR FAMILY MEMBER.

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Zinc is an essential nutrient due to its role as a structural co-factor for protein folding and as a catalytic co-factor for many enzymes. However, if this nutrient accumulates over a given threshold, it can become toxic to the cell. For these reasons, it is absolutely critical for cell survival that zinc homeostasis be tightly controlled. In *Bacillus subtilis*, a model Gram positive organism, the response to zinc limitation is mediated by Zur which acts as a classical repressor when Zinc is present. The Zur regulon has been characterized and currently contains ten genes all suspected or known to contribute to the zinc starvation response. Classically this response was thought of in terms of obtaining external zinc by high affinity pumps. In part, the work presented within will show that our understanding bacterial metal ion homeostasis is expanding. We now understand that in addition to uptake as a metal limitation response, cells also create duplicates of zinc requiring proteins which have altered metal co-factor specificity, a mechanism we classify as substitution. Finally, bacteria use mobilization as a response mechanism by creating conditions in which zinc containing proteins are replaced and the zinc which they contained is used for continued growth in zinc limiting conditions.

The work presented here shows a cross-section of the molecular mechanisms Zur employs to achieve zinc homeostasis in *Bacillus subtilis.* Starting at the protein biochemistry level, I will present studies aimed at addressing how Zur senses zinc.

This work uses a site directed mutagenesis approach to identify amino acid residues which make up the three conserved zinc binding pockets within the Zur protein and how each contributes to Zur's function. Second, after understanding how the protein senses zinc, I sought to understand how Zur regulated one of the uncharacterized members of its regulon, the *yciC* gene. In addition to elucidating the regulation of the unique promoter structure of *yciC*, I also show that Zur binds a consensus 9-1-9 inverted repeat. Finally at a physiological level, I studied the three ribosomal genes under the control of Zur to understand their contribution towards the zinc starvation response.

## BIOGRAPHICAL SKETCH

Scott E. Gabriel was born in Columbus, OH while his father was finishing his PhD at Ohio State University in Genetics. Upon his father's successful defense, this new family moved to Williamsport, PA where the students and faculty of Lycoming College became extended family for Scott's childhood years and his love of science and the small liberal arts college environment germinated. The college shaped many of his early memories: show and tell day meant a trip to the biology stockroom; occasional Saturdays were spent helping his dad correct tests or visiting his office to see the salt water aquarium and the bee hive; and Sundays students were frequently invited over to our home for waffles or to join us on our family hikes. After graduating high school, Scott majored in Biological Sciences at the University of Pittsburgh where he worked in the lab of Dr. Karen Arndt studying the formation of the yeast pre-initiation transcription complex. Upon obtaining his B.S. degree, he joined the Coalition for Christian Outreach, a regional campus ministry organization and accepted the position of Assistant Chaplain at Ohio Wesleyan University in Delaware, OH. At Ohio Wesleyan, Scott mentored students by using the outdoors as a classroom. He led over 50 wilderness trips and accumulated over 100,000 contact hours leading and teaching students in the wilderness. In the Fall of 2004 Scott resigned at Ohio Wesleyan to pursue his PhD at Cornell University with the goal of returning to a small liberal arts college as a professor. In the summer of 2005 Scott joined John Helmann's laboratory because of his long held fascination with the subject of transcription regulation. His research has focused on how *Bacillus subtilis* senses and responds to metal ion limitation. Upon the completion of his doctorate degree, Scott and his family will be relocating to La Crosse, WI where he will join the faculty of Viterbo University teaching Biochemistry.

This thesis is dedicated to my family and to our shared
memories which create a life well lived..

to Sunday family hikes, grilled cheese sandwiches, a fledgling bottle return business, weeding and the labor department, summers filled with salt water, flying fishing poles, kings in the corner, summer whiffle ball games with the cousins, lobster dinners on July 6, the Old Mill Inn, Sunday waffles, labor days at the camp, canoe trips, family lake nights, walks with rocks for Toby, Catan, St. Petersburg and may other game filled nights, cross-country skiing on the North shore, curvy roads in Canada, house boats with slides, little boys who come six weeks early, sleepless nights, and toothless grins.

And to all of the memories yet to be written…
I love you all and am grateful to call each of you family.

Scott

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# CHAPTER 1

## METAL ION HOMEOSTASIS

The variability of nutrients, temperature, moisture, and toxins that *B. subtilis* must face in its natural soil environment requires that this organism have an efficient response network to neutralize toxic compounds, efflux excess nutrients and scavenge for limiting ones. At the most fundamental level, these required nutrients, are or are comprised of the small fraction of the known elements which biological life uses. In fact, 99.9% (wet weight) of a cell is comprised of ten elements: carbon, hydrogen oxygen, nitrogen, sodium, potassium, calcium, magnesium, phosphorus, and sulfur (35). While the components of the remaining fraction might seem insignificant, this could not be further from the truth. These trace metals (Fe, Mn, Zn, Cu, Ni, and Co) are involved in some of the most essential cellular processes both as structural and catalytic co-factors (3). In fact it is estimated that up to one third of all proteins and almost half of all enzymes require a metal cofactor for proper function (3, 28). However as important as trace metals are, in excess they become toxic to cells and therefore it is not surprising that their uptake and efflux are strictly regulated (70, 75).

This chapter outlines the topic of metalloregulation with specific focus on the response to metal ion limitation and will serve as context and background for the work presented within on zinc homeostasis in *B. subtilis*. Specifically, the following questions will frame the exploration of three increasingly complex levels of metalloregulation: First, at the elemental level what are the absolute and biologically available amounts of the trace metals inside the cytosol and what role do they play in the cell? Second, at the protein level how do metalloregulatory proteins sense metals and alter gene expression? And finally, at an organismal level what mechanisms are employed in response to metal ion limitation?

## 1. The Elements

Central to the model of homeostasis is the concept of a set point at which the organism is satisfied with the levels of each given metal. Determining at what metal concentration uptake and efflux responses are initiated helps us to define the normal intracellular levels of each specific metal. Recent advances in technology such as inductively coupled plasma mass spectrometry (ICP-MS), X-ray fluorescence microscopic imaging, and metalloproteomics (104), are also aiding our understanding of metal homeostasis and guiding us towards a more systematic view of metal location and levels inside cells (90, 91). While only a few organisms have been subjected to such a thorough accounting of the "metallome"(34, 80), recent evidence suggests that the relative molar abundance of trace metals may remain constant over a diversity of bacterial species (8). However, several studies have also shown that absolute cytosolic levels of trace metals are not standardized across species (15, 43).

A comparison of the trace metals (Mn, Fe, Co, Ni, Zn, Cu) reveals their structural similarity: they all have a double positive charge, as well as very similar ionic diameters (106). For cells to unequivocally discriminate between the metals they encounter would require them to tightly bind the metal, which is an energy consuming process (73). To avoid this energy sink, cells possess generally two distinct uptake systems. The first is fast, non-specific, and constitutively expressed which uses energy from chemiosmotic gradients to drive uptake of a wide range of similar metals across the membrane (73). The second is a time and energy expensive investment involving ATP driven pumps which allow for specific and high affinity uptake of one given metal (74). Due to the energy cost of such a system, their expression is tightly controlled by metalloregulators and they are only produced in times of starvation. The following section will examine the roles of each of these transition metals, their cytosolic concentrations, and the coordinated regulation which achieve these levels.

## 1.1 Manganese

The metal manganese has a generally low toxicity to cells, since it has a low affinity towards thiols, is unlikely to replace other transition metals from their binding sites and very rarely forms radicals. In fact, manganese supplementation has been shown to help several bacterial species cope with oxidative stress, which otherwise would have been lethal (24, 89, 98). Until very recently, it was thought that manganese was able to protect against oxidative stress through chemical scavenging (46), however work in *E. coli* suggests protection against oxidative damage is mediated by replacing $Fe^{2+}$ in iron-containing proteins with $Mn^{2+}$ (5). This finding explains the complex regulation of the manganese uptake transporter, MntH, in *E. coli*. The *mntH* promoter region contains operator sites for both the $H_2O_2$ responsive OxyR regulator and iron homeostasis regulator Fur (53). A similar regulatory connection between manganese uptake and oxidative stress response is found in *Salmonella* (52). In contrast, in *B. subtilis* MntR, a Mn(II) specific DtxR like repressor, regulates the expression of both the low affinity transporter (MntH, a homolog of the natural resistance-associated macropage protein (NRAMP) which uses a proton coupled metal transport method (16, 17)) and the high affinity uptake system (MntABCD , a ABC transporter (9, 10)) in response to manganese levels irrespective of oxidative stress (44, 84).

Although it seems not to be involved in protection against oxidative stress in *B. subtilis*, manganese is known to be important for sporulation with an increase in manganese uptake occurring upon entering stationary phase (29). Additionally manganese is used by a diversity of enzymes including many transferases, peptidases, hydrolases, and enzymes involved in metabolism (21, 109).

While intracellular levels of manganese can change in response to growth phase (i.e. stationary), nutrient availability, or encountered stress (i.e. oxidative stress),

rough estimates of intracellular manganese concentrations have been proposed. As a first estimate total cellular manganese has been measured at 10μM in rich media for *E. coli* (80). However the level of "free" manganese is likely to be slightly lower once the proteins which bind Mn(II) as a co-factor are accounted for. One approach to defining intracellular levels of metals is to determine the affinity of the respective metalloregulator for its relevant co-factor. *B. subtilis* MntR is estimated to bind manganese at a $K_d \approx 150\mu M$ (41) and the *Rhizobium leguminosarum* Mur, a Fur family Mn(II) metalloregulator, was found to have micromolar affinity for Mn(II) as well (12). Due to this relatively low affinity, the current hypothesis is that the selectivity of these regulators for Mn(II) is imposed by the geometry of the binding pocket or the action of a metallochaperone (12, 41). Alternately it remains possible that intracellular manganese levels persist between 50-100μM and the observed metal constants are relevant *in vivo*. Support for this last option is provided by NMR studies in *Staphylococcus aureus* which determined that the intracellular levels of manganese are in the range of 50-100μM (31). In *Borrelia burgdorderi*, which grows in an iron starved environment, manganese levels are 2.5 fold higher when compared to *E. coli* seemingly as an adaptation to the local metal availability (83). While in L*actobacillus*, which grows on Mn-rich plant materials, intracellular manganese levels reach as high as 30mM (6). However, since these measurements exist for only a small fraction of bacteria, it remains unclear what range if any is the norm.

**1.2 Iron**

Iron is the most abundant of the transition elements in bacteria (8). This likely is due to the vast diversity of roles it plays in the function of the cell. Iron acts a co-factor for enzymes involved in respiration (both aerobic and anaerobic), photosynthesis, DNA synthesis, nitrogen fixation, TCA cycle, and metabolism (4, 35). Critical to acting as a co-factor for many of these enzymes is its ability to exist is two

oxidation states: the ferrous ion ($Fe^{2+}$) and a ferric ion ($Fe^{3+}$). When found in aerobic environments with neutral pH, iron is oxidized to its ferric state which is sparingly soluble in aqueous solution ($10^{-18}$M). In general bacteria require total iron levels to be between $10^{-7}$ to $10^{-5}$M to achieve optimal growth (4) and in agreement with this finding, total iron content in *E. coli* was measured to be approximately $10^{-4}$M (80). Given this gap between iron availability and nutritional requirement, it is not surprising that bacteria spend a considerable amount of energy acquiring and transporting iron. Bacteria use as many as five separate mechanisms for iron acquisition employing a host of transport proteins with varying functions which are commonly under the control of Fur, the iron starvation response regulator. In *B. subtilis*, Fur controls approximately 40 genes, the vast majority of which are involved in siderophore biosynthesis and uptake (70, 79). Siderophores are low molecular weight Fe(III) chelators widely used by bacteria with over 500 currently identified (4). Once synthesized, siderophores are secreted, they scavenge environmentally available ferric iron, and are then recognized by membrane-bound binding proteins and transported into the cell by ATP hydrolysis. *B. subtilis* synthesizes the siderophore bacillibactin whose uptake into the cell is dependent upon the FeuABC transporter, also a member of the Fur regulon (68, 79). Once transported into the cell an esterase, YuiL, releases the bound iron (68). While only one bacillibactin is synthesized, *B. subtilis* possesses several substrate binding proteins able to recognize siderophores produced by other organisms (71). In addition to iron uptake through siderophores, *B. subtilis* also contain an elemental iron uptake system and a iron-citrate transporter (79). In pathogenic bacteria such as *Nisseria* and *Haemophilus* species members, this acquisition repertoire is expanded by the presence of receptors able to bind and remove iron from host proteins including heme and transferrin compounds (4, 23, 39).

Once iron is taken up, the reducing environment of the cell allows iron to remain in its ferrous state. As mentioned previously, total iron levels in the cell approach mM levels due to the amount of iron containing proteins in the cell. Once iron is incorporated into heme or Fe-S centers of proteins, there must remain an available pool of iron which continues to metallate proteins, aid in transport and storage of iron, and bind to iron regulatory proteins. The question is, at what concentration is this pool maintained in the cell? One risk in having free iron is its redox potential and ability to react with $H_2O_2$ in Fenton chemistry resulting in DNA and protein damaging free radicals. Several experimental approaches have provided data suggesting that iron is maintained at the tens of micromolar range. EPR studies in *E. coli* have reliably determined that normal wild type levels of free iron are 10-30μM (54, 108). This is further supported by the finding that *E. coli* Fur has a 1.2μM affinity for iron (69). However free iron levels can rise significantly when cells encounter oxidative stress due to the release of iron from damaged Fe-S centers (54).

**1.3 Cobalt & Nickel**

The importance of both cobalt and nickel in biological processes has decreased significantly with the advent of an aerobic environment. In the early electron-rich atmosphere where metabolism of single carbon compounds would have been critical, both nickel and cobalt would have been essential (33). Currently there are only a handful of enzymes that still require these two metals as co-factors. For nickel there are nine identified enzymes: a characterized family of Ni-SOD's which seems to be used in bacteria found in iron-limiting conditions (27), NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl CoA-decarbonylase/synthase, methyl coenzyme M reductase, some glyoxylases, aci-reductone dioxygenase, methylenediurease and most notably urease (72). Nickel is also a component of factor F450 found in methanogens (26). Many of these enzymes are only used by a handful of organisms and can often be

replaced by paralogous enzymes containing alternate metal co-factors. Interestingly urease, the only known nickel requiring enzyme in *B. subtilis* is missing the accessory factors required to metallate the enzyme possibly alluding to the usage of additional metals (55). In contrast cobalt is only sparingly used in microbial metabolism serving mainly as a component of coenzyme-$B_{12}$ and its associated reactions as well as a co-factor for methionine amino peptidase (56).

Given the relatively limited roles these metals play in cellular metabolism and the fact that cobalt can damage Fe-S centers by displacing iron (86), it is expected that they would be found in low amounts within the cell. Uptake of both $Co^{2+}$ and $Ni^{2+}$ in *E. coli* are thought to occur surreptitiously through the CorA magnesium transport system (which was actually first identified by a cobalt resistance phenotype (107) as well as other divalent pumps such as ZupT (42) or NRAMP (See figure 1-1 and (1)). Additionally in *E. coli* and other bacteria, nickel can be imported through a high affinity uptake system (NikABCDE) which is controlled by a nickel responsive regulator NikR (19, 20) or the widely distributed NiCoT family involved in cobalt and nickel uptake (87). Interestingly, none of these uptake systems are conserved in *B. subtilis*, alluding to a possible lack of requirement for cobalt and nickel.

**1.4 Zinc**

Zinc is second only to iron in absolute abundance among the trace metals in biology. Zinc is also the second strongest Lewis acid in the cell following copper. However, in contrast to both iron and copper, zinc is non-redox active under physiological conditions due to its filled *d* shell. At the intersection of these three characteristics of zinc lies the explanation for the many diverse ways proteins utilize zinc as a cofactor. A recent survey of zinc binding proteins found that they comprised 4-10% of an organisms' entire proteome and that the number of zinc-binding proteins correlates linearly with the total number of proteins encoded in an organism (2). For *B.*

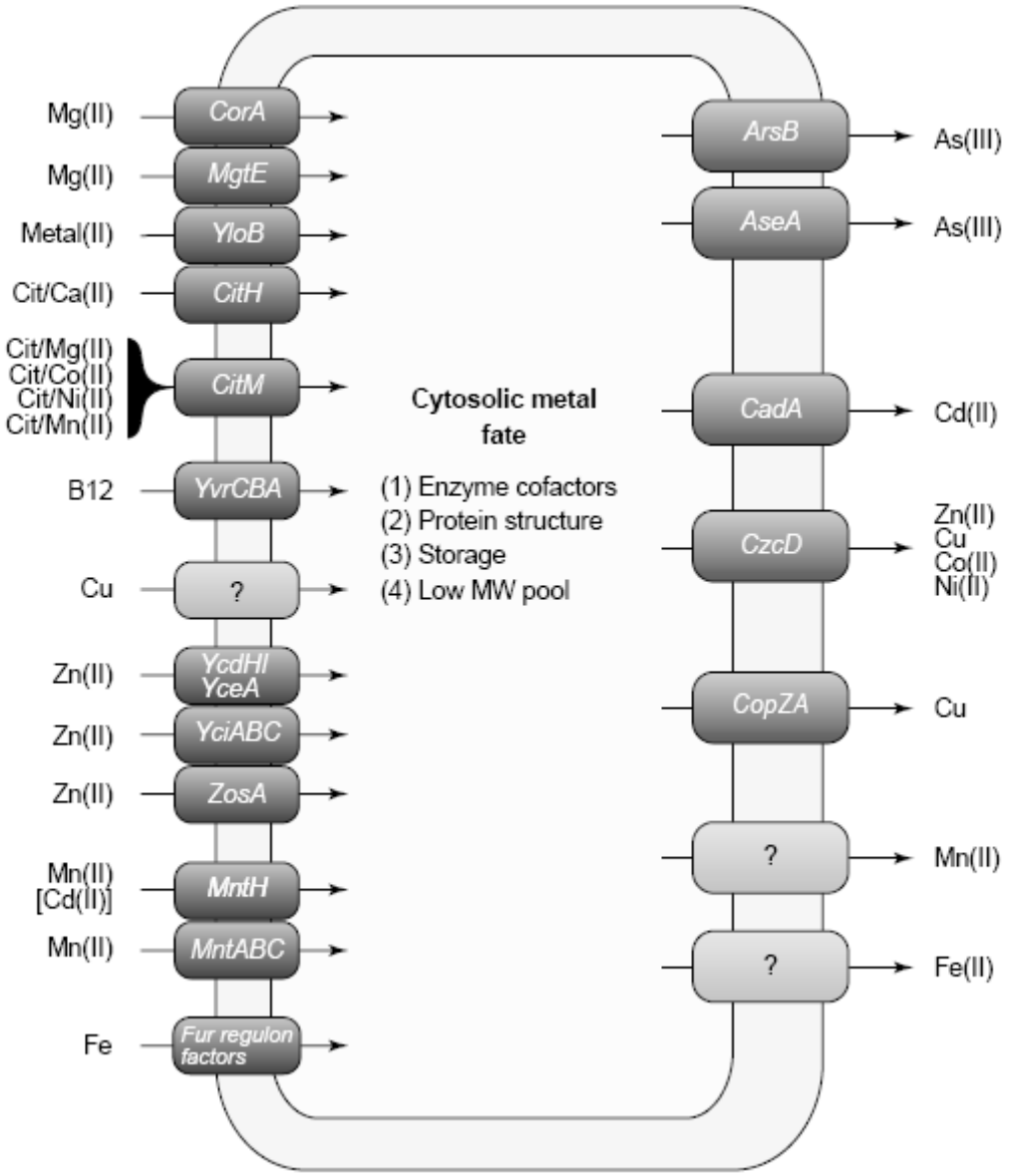


Figure 1.1 Metal homeostasis systems in *B. subtilis*. Uptake system on the left and export system on the right. Taken from (71)

*subtilis* these bioinformatics studies have predicted over 200 zinc-containing proteins or 6% of the proteome (2). A survey of these identified proteins reveals enzymes involved in DNA synthesis, repair, and regulation as well as protein synthesis, general metabolism and stress responses all require zinc (2).

Given the sheer volume of proteins which use zinc as a co-factor, we would expect a fairly high total zinc quota for the cell. In *E. coli* this number has been estimated to be 200μM (80). In this same study, the homeostatic levels of free zinc within the cell were determined by examination of zinc metalloregulators for uptake and efflux. The half maximal point of Zur induction occurred at $10^{-16}$M and the half maximal induction point of zinc efflux mediated by ZntR occurred at $10^{-15}$M (80). Work presented within is consistent with a similar femtomolar level of free zinc in *B. subtilis*. Having essentially no free zinc within the cell, and a multitude of zinc-requiring enzymes, raises the question of how zinc requiring proteins and enzymes are metallated. Many researchers have suggested a metallochaperone may function in this role, but to date no zinc chaperone has been identified in any organism.

**1.5 Copper**

Copper is most frequently found in one of two oxidation states: its cupric form (Cu(II)) or its reduced cuprous form (Cu(I)) and constitute the most effective divalent and monovalent ions respectively for binding to organic molecules that are available in biological system (35). This avid binding affinity for biological ligands risks the displacement of other cognate metals cofactors thus altering protein function. Additionally, copper has a strong ability to catalyze Fenton chemistry. Both of these reasons provides support to the suggestion that there is no need for copper to be transported into the cytosol (94). In *B. subtilis* Cu(I) levels are regulated by CsoR, a representative of a newly discovered family of metal responsive DNA binding regulators (CsoR/RcnR family) (50, 92). Characterization of the metal binding affinity

of this regulator determined greater than zeptomolar affinity for Cu(I) (63), similar to the previously reported affinity of the *E. coli* CueR copper regulator (18) providing further evidence that there is no free copper inside the cell. In *B. subtilis* CsoR regulates the *copZA* operon (Fig 1-1) which encodes a copper chaperone (CopZ) and a copper efflux P-type ATPase (CopA) (92). These proteins ensure that any copper that is found in the cytosol can be safely transported to the efflux channel. Although copper is apparently excluded from the cytosol, *B. subtilis* does express two copper-requiring enzymes: a *caa3*-type cytochrome oxidase, located at the cytoplasmic membrane (100), and a copper laccase (CotA) located in the spore coat (48). Unlike *Enterococcus hirae* which encodes a dedicated high affinity copper uptake system CopA (76, 78), no dedicated copper uptake system has been identified in *B. subtilis* and it is likely that copper enters the cell through the CorA-$Mg^{2+}$ channel.

**2. The Proteins**

The measured intracellular levels of free metals discussed in the previous section are predicated on a finely tuned system of metalloregulatory proteins, uptake and efflux channels, metallochaperones, and a cellular milieu which binds and buffers many metals. In the infancy of metalloregulation, it was naively assumed that each regulator would selectively bind its metal co-factor out of this cellular milieu based only on its affinity. Robinson and coworkers have posited that affinity is only factor in the ability of proteins to achieve selective responses to metal availability: allosteric changes as a result of the binding interaction and the ability to access the metal are just as vital (45, 94, 96).

Seven families of bacterial metalloregulatory proteins have been identified (Table 1.1). These families can most readily be divided into those which are responsible for uptake of essential metals (Fur, DtxR, and NikR) and those which

efflux toxic metals (MerR, ArsR, CsoR, and CopY). While traditionally this distinction has held true, it is becoming blurred with a recent example of Zur from *X. campetris* regulating both uptake and efflux (47). In *B. subtilis* representative members from five of the families have been identified thus far (Fur, DtxR, MerR, ArsR, CsoR). The following section will discuss recent examples highlighting how metalloproteins distinguish their specific co-factor and relate these findings to our current understanding of the Fur family members in *B. subtilis.*

For details of the contribution of each of the metalloregulatory proteins to metal ion homeostasis the reader is directed to the following recent reviews (40, 71, 103).

**2.1 Affinity**

Despite the diversity of metals within the cell, metalloregulatory proteins bind their cognate co-factor with great specificity (82). This specificity can only be partially explained by the binding affinities of these proteins for their intended metal co-factor. Generally metalloregulators have high affinities for their intended metal co-factor and in turn control a matching transcriptional response. For example, MerR's sensitivity to $Hg^{2+}$ and its regulation of mercury resistance genes or Zur's femtomolar binding of $Zn^{2+}$ and the coordinated regulation of zinc uptake. However, the Irving Williams series (49) predicts that if metal binding was determined solely by affinity, there would be only copper and maybe zinc metalloproteins in the cell. This series describes divalent metals tendency to display a conserved binding order when binding to proteins ($Mg^{2+}$, $Ca^{2+}$ <<<$Mn^{2+}$ < $Fe^{2+}$ < $Co^{2+}$ < $Ni^{2+}$ < $Cu^{2+}$ > $Zn^{2+}$). Indeed this holds true in numerous cases as exemplified by the manganese regulator MntR, which controls the two manganese transporters MntA and MntH. However, MntR actually binds several metals ($Ni^{2+}$, $Zn^{2+}$, and $Co^{2+}$) *in vitro* with greater affinity than its native co-factor $Mn^{2+}$ (41). Another similar example is the *E. coli* NikR protein that has been shown to have significant affinity to several metals ($Co^{2+}$, $Zn^{2+}$, $Cd^{2+}$ and $Cu^{2+}$) in

Table 1.1 Overview of Families of bacterial metalloregulatory proteins

| Family | Representative members[1] | Metals (metalloids) sensed[2] |
|---|---|---|
| Fur | Fur, Zur, Mur, Nur, Irr, (PerR) | $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ni^{2+}$ |
| DtxR | DtxR, IdeR, MntR, ScaR, SirR | $Fe^{2+}$, $Mn^{2+}$, ($Zn^{2+}$), ($Cd^{2+}$) |
| NikR | NikR | $Ni^{2+}$ |
| MerR | MerR, ZntR, CueR, PbrR, CadR | $Hg^{2+}$, $Zn^{2+}$, $Cu^{1+}$, $Pb^{2+}$ |
| ArsR | ArsR, SmtB, CadC, CzrA, NmtR | $As^{3+}$, $Bi^{3+}$, $Zn^{2+}$, $Cd^{2+}$, $Pb^{2+}$, $Co^{2+}$ |
| CsoR | CsoR, RcnR | $Cu^{1+}$, $Ni^{2+}$, $Co^{2+}$ |
| CopY | CopY | $Cu^{1+}$ |

[1] Note that PerR is not considered a metalloregulatory protein by the criteria used here.
[2] Metals where the physiological relevance of the induction is uncertain are in parentheses

addition to its relevant *in vivo* co-factor $Ni^{2+}$ (105). Additionally, several of these non-cognate metals mediated significant DNA binding affinity by NikR (13). These few examples emphasize the requirement for more than just affinity to drive the metal binding properties of proteins.

While the three Fur paralogs in *B. subtilis* (Fur, Zur, PerR) (14, 38) have not been characterized to the level described in the previous examples, a related problem is revealed when their protein sequences are aligned. These proteins exhibit three very similar conserved metal binding sites, yet these proteins sense $Fe^{2+}$, $Zn^{2+}$ and peroxide stress respectively. The biochemical mechanisms by which this common motif can be utilized for such a wide variety of co-factors remain to be elucidated.

**2.2 Access**

As previously mentioned in section 1.4 and 1.5, intracellular metals such as zinc and copper are highly buffered such that the free metal at equilibrium is very low. By limiting the amount of the metals at the top of the Irving Williams scale, cells enable other weaker-binding metals to associate with their cognate metalloproteins. Restricting the levels of free metal inside the cell forces the metalloproteins to compete for a limited supply of available metals, and therefore the relative affinity of the protein for the metal becomes more crucial. While this concept of access dictates many of the metal and protein interactions inside the cell, one particular example of protein folding localization highlights this concept well. Tottey and coworkers set out to determine how proteins which use metals at both ends of the Irving Williams series are produced, fold and are metallated within a common cytosol (96). They isolated the most abundant $Cu^{2+}$ and $Mn^{2+}$ containing proteins of the *E. coli* periplasm (CucA and MncA respectively) and to their surprise they found that both CucA and MncA contained a cupin fold which bound their respective metal co-factors. However, most significantly the proteins varied in their mode of export. MncA is secreted by the Tat

pathway allowing the protein to fold and to be metallated in the cytoplasm where high levels of $Mn^{2+}$ are present. However CucA is secreted by the Sec pathway so that it folds and is metallated in the periplasm where it can acquire $Cu^{2+}$ (96).

Especially in the cases of zinc and copper where essentially no free metal is accessible, it is presumed that chaperones mediate access to these metals. As mentioned previously, there is to date no known zinc metallochaperone, however a few copper chaperones have been identified (77, 85, 95). In *E. hirae* copper uptake is mediated by the ATPase CopA which transfers copper to the metallochaperone CopZ. Expression of this system is controlled by the DNA binding metalloprotein CopY. As evidence that metallochaperones mediate access, deletion of *copZ* impaired CopY's ability to sense copper and to regulate a promoter fusion reporter and this direct interaction has been confirmed with *in vitro* using purified proteins (22, 77).

**2.3 Allostery**

Metalloregulation requires the action of metal-binding to induce a structural change in the protein which results in modulated DNA-binding and a subsequent transcriptional response. This cascade of events is the subject of this last section in addressing the mechanisms by which proteins discriminate metal binding.

*M. tuberculosis* NmtR, an ArsR family member, mediates resistance to $Ni^{2+}$ and $Co^{2+}$. However, *in vitro* NmtR binds $Zn^{2+}$ with the greatest affinity even though it does not mediate $Zn^{2+}$ resistance (15). Alternately, SmtB, also an ArsR family member, does provide $Zn^{2+}$ but not $Ni^{2+}$ resistance in *M. tuberculosis*. Mutagenesis studies performed on these two regulators suggested that NmtR coordinated metals via six binding residues while SmtB only used four (15, 99). Examining metal binding by UV spectroscopy showed that $Co^{2+}$ bound in an octahedral geometry in NmtR, but in a tetrahedral geometry in SmtB (15). Using X-ray absorption, Zn was found to bind NmtR in a tetrahedral geometry. The authors conclude that it is this octahedral

geometry of metal binding which is required for the allosteric switch to occur in NmtR allowing derepression (15). So even if $Zn^{2+}$ binds NmtR *in vivo*, it does not induce a conformational change. Further work elucidated that only when bound in the preferred geometry did a hydrogen bond network form altering the structure of the protein and thus resulting in DNA regulation (82).

While the details of mechanism are not understood to this depth, this theme of allostery holds true for many other examples. *B. subtilis* MntR as mentioned previously binds several metals which are not related to its function inside the cell, as does both *E. coli* and *B. subtilis* Fur, and *E. coli* NikR. In all of these instances it is likely only the relevant metal co-factor that causes the cascade of interactions which induces the allosteric change resulting in a protein conformational change.

In the case of the Fur family members, many questions remain regarding the roles of the metal-binding sites and even in some case the identity of the relevant *in vivo* metal co-factor (7, 25, 69). The best biochemically characterized Fur family member is *B. subtilis* PerR which senses hydrogen peroxide(14). Lee and Helmann determined that site 2 of PerR can bind either $Mn^{2+}$ or $Fe^{2+}$ as a metal co-factor (36, 61). When PerR is bound with $Mn^{2+}$ this form is highly insensitive to oxidative stress. However when site 2 is bound with $Fe^{2+}$ a metal catalyzed oxidation occurs (61). Oxidation of the histidine(s) in this metal binding site induces a conformational change in PerR releasing its repression of its regulon and resulting in a peroxide stress response. The structures for both Apo-PerR (97) and metal bound PerR (51) have been solved and when compared show the drastic allosteric change in conformation which allows this protein to bind DNA.

**3. The Organism**

The metals and proteins which have dominated the discussion to this point serve as components of a stress response system which allows the organism to respond

appropriately to environmental stimulus and thus survive deleterious conditions. It is the creative nature of these stress responses orchestrated by the metalloregulatory and other proteins which has enabled bacteria to tolerate the extreme ranges of niches in which they are found.

This chapter started with the statement that 99.9% of all biological life is comprised of ten elements with the remaining portion accounted for by the transition metals. We have discussed the most important of these transition elements, cataloged some of the enzymes in which use them as co-factors and detailed the metalloproteins which sense their limitation or excess. The question we will end with is: what is the nature of these response mechanisms triggered upon nutrient limitation?

The most ubiquitous response to metal limitation is to create high affinity transporters. While uptake is an important response, what if there is no metal to be taken up? There must be other limitation responses in place to complement the creation of transporters. My work described within, along with a growing body of literature suggest at least two additional mechanisms help organisms respond in times of nutrient limitation. This section will focus on the recently appreciated responses to limitation of elemental sparing and elemental substitution.

**3.1 Elemental sparing**

The most prominent example of this class is the iron-sparing response first described in *E. coli* which is controlled by the iron responsive regulator Fur (65). When iron is sufficient, Fur acts as a transcriptional repressor, however when iron becomes limiting Fur becomes inactive and allows the expression of genes controlling Fe(II) uptake pathways and also the sRNA RhyB. RhyB has been shown to bind to mRNAs that encode protein which utilize iron and cause their degradation through the action of RNAaseE (64). By this mechanism, the limited cellular iron supply can be redirected to essential iron requiring proteins rather than diverted into nonessential

iron-utilizing proteins. Currently there are over 50 genes which have been shown to be regulated by this sRNA iron sparing response (66). An analogous sRNA iron sparing system has recently been characterized in *B. subtilis* also under the control of Fur (37). Several of the confirmed targets of the *B. subtilis* sRNA are analogous to RhyB action in *E. coli*, however significant differences do exist and the investigations into the breadth of the regulation of this response are still in their infancy.

Another example is a sulfur sparing response initiated by yeast in response to cadmium stress (32). In yeast glutathione (GSH), a sulfur containing tri-peptide, is induced in response to cadmium stress constituting an important component of intracellular cadmium detoxification system (62). Upon sensing cadmium stress, a sizable pool of sulfur (30% of the entire sulfur pool) is diverted towards GSH synthesis. This adaptation is achieved by producing isozymes containing fewer Met and Cys amino acids than their counterparts (32, 102). Interestingly, this adaptation targets the most abundant proteins, regardless of their activity in the cell thereby achieving the greatest savings of sulfur for the cell (32). In a related examples, when *Salmonella typhimurium* encounters sulfur-limiting conditions, it abundantly produces a sulfate permease which lacks both Met and Cys residues (81). While cyanobacteria create sulfur depleted version of their most abundant proteins upon encountering sulfur limitation (67).

On a larger scale, the model organisms *S. cerevisiae* and *E. coli* were used to test the hypothesis that nutrient starvation might affect the evolution of the proteins involved in the acquisition of the starved nutrient (11). The atomic composition of carbon and sulfur assimilation proteins were compared with that of the remaining proteome of these organisms. Addtionally, nitrogen starvation was examined in just *S. cerevisiae.* In all cases studied, carbon, nitrogen and sulfur atoms were found to be decreased in the subset of proteins involved in the assimilation of these elements

during starvation conditions as compared to the average occurrence of these elements at the proteome level (11).

### 3.2 Elemental substitution

In addition to having mechanisms which help redirect limiting nutrients to the most critical cellular functions, organisms have also found ways to substitute alternate metal co-factors to replace those in limited abundance. One of the earliest observed examples of this type of stress response occurs in the cell wall of *B. subtilis.* Under phosphate-limiting conditions, the phosphate containing anionic polymer techoic acid is replaced with teichuronic acid, which contains only one phosphorus atom (58). The relative ratios of these two anionic polymers were found to correlate directly with phosphate levels in the media (57, 60). Interestingly regulation appears to occur at the level of incorporation of the two polymers into the cell wall. Upon sensing phosphate limitation, only teichuronic acid is incorporated. Although techoic acid is still synthesized, it is secreted instead of being incorporated (57).

Another example of substitution in response to phosphorous limitation is found in cyanobacteria who have evolved to use sulfur instead of phosphorous in their lipids (101). Therefore these organisms are unaffected by the drastic fluxes in levels of dissolved phosphorus in the open ocean and possess a competitive advantage over other bacteria that lack this adaptation. Additionally in cyanobacteria, iron is widely used in many of the photosynthetic pathway enzymes. One of these proteins, ferredoxin is an Fe-S containing protein involved in electron transport in the chloroplasts. When these organisms encounter iron starvation conditions ferredoxin can be functionally replaced with flavodoxin (93). While this example is not a strict metal for metal substitution, the final outcome of being able to endure the nutrient limitation stress encountered is similar.

Two final examples of this mechanism focus on zinc limitation conditions. First a cadmium-dependent carbonic anhydrase (CA) enzyme has been isolated from a marine diatom, whose natural environment contains very low amounts of trace metals (59). This is the first report of a $Cd^{2+}$ dependent CA, classically a $Zn^{2+}$ dependent enzyme, and provides another example of adaptation to nutrient limitation. Another $Zn^{2+}$ requiring enzyme is GTP cyclohydrolase (GCYH). This enzyme is essential for folic acid biosynthesis in bacteria, plants, and fungi. Recently it was shown that several genomes contain a functional duplication of this required enzyme (30) and in follow up work it has also been shown that this duplicated protein does not require $Zn^{2+}$ (88). In *B. subtilis* this duplicate enzyme is under the control of Zur and is expressed during zinc limitation. Our recent findings show that this enzyme does not require $Zn^{2+}$ ,but is active with $Mn^{2+}$ and/or $Fe^{2+}$ (88).

**4. Concluding remarks**

Metal ion homeostasis can be defined as controlling intracellular metal concentrations. The simplicity of the given definition would betray the depth of complexity which is undertaken by a single cell organism to achieve such a goal. In this chapter I have attempted to give a cross section of the levels involved in achieving metal ion homeostasis in bacteria. Each part alone does not comprise the whole: it is not just a matter of elements their chemical properties and biological relevance; nor is it a matter of proteins and the mechanisms by which they bind varying metals; nor is it just a matter of the observed stress responses, but the complex network built by the intersection of all of these areas.

The following chapters will attempt to mirror this cross-section presented by specifically looking at zinc homeostasis in *B. subtilis*. The first chapter focuses on a site directed mutagenesis approach to identify amino acid residues which make up the

three conserved zinc binding pockets within the Zur protein and how each contributes to Zur's function. Second, after understanding how the protein senses zinc, I sought to understand how Zur regulated one of the uncharacterized members of its regulon, the *yciC* gene. In addition to elucidating the regulation of the unique promoter structure of *yciC,* I also show that Zur binds a consensus 9-1-9 inverted repeat. Finally at a physiological level, I studied the three ribosomal genes under the control of Zur to understand their contribution to the zinc starvation response.

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# CHAPTER 2

# BIOCHEMICAL CHARACTERIZATION OF THE THREE CONSERVED METAL BINDING MOTIFS OF *BACILLUS SUBTILIS* ZINC UPTAKE REGULATOR (ZUR)

## 1. Summary

*Bacillus subtilis* Zur (BsZur), a Fur family member, upon sensing zinc starvation allows the transcription of a ten gene regulon. While work has been done to characterize Zur regulon members, the metal binding site(s) responsible for sensing zinc by Zur remain unclear. To date there are five metallated Fur family protein crystal structures published which have visualized as many as three distinct metal binding sites in this family of proteins.

In this study we investigated the functions of these three proposed Zur metal binding sites and their affects on BsZur's activity as a transcriptional regulator. Based on sequence homology alignment and modeling with published crystal structures, we created a suite of different site mutants to target residues involved in metal binding. Those mutants exhibiting the most significant altered repression patterns *in vivo* were further characterized biochemically. Our work confirms that Zur senses zinc with femtomolar affinity at its sensing site and additionally is stabilized by zinc binding at the $Cys_4$ structural site motif both comparable to its *E. coli* homolog. Additionally, we show Zur binds a total of two zinc atoms per monomer. However it remains unclear if the second zinc is bound exclusively at site 1or 2 or is capable of binding at either site. Finally, we present the initial characterization of site 1 and 2 double mutants which suggest that sites 1 and 2 of BsZur may play compensatory roles.

## 2. Introduction

Many essential proteins require zinc as a catalytic and/or structural metal co-factor (4). However when in excess zinc can become toxic (18). Therefore controlling intracellular levels of this metal is of the utmost importance. In *Bacillus subtilis*, a model Gram positive microorganism, Zur regulates genes which respond in times of zinc limitation. The Zur regulon, as currently defined, contains a high affinity zinc uptake transporter (*ycdHIyceA*) homologous to the ZnuABC transporter of *E.coli*, a non Zn-requiring GTP cyclohydrolase (*yciA*), a proposed metallochaperone (*yciC)*, three ribosomal protein paralogs (*ytiA, yhzA, rpmGC*), and a gene of yet unknown function (*zinT*) (7, 10).

The current model for the transcriptional repression of these ten genes is that when bound with zinc at its sensing site, Zur undergoes a conformational change enabling it to bind DNA thus repressing transcription. When zinc becomes limiting in the cell Zur's sensing site is no longer bound with zinc and therefore dissociates from its operator site. This operator site has been characterized for the BsZur protein and optimally contains a 9-1-9 inverted repeat which is slightly larger then the conserved Fur operator sequence (11).

While this mechanism of regulation is well documented for Fur family members (5, 6, 9, 25), and likely the most prevalent among these transcriptional regulators, there is an increasingly number of examples where Fur family regulators have been shown to act as transcriptional activators. For example, in *Caulobacter crescentus* Fur acts a repressor of iron uptake proteins, but also directly activates iron-utilizing enzymes (6). Additionally, Zur in *Xanthomonas campestris* also possesses dual functions as an activator and repressor of transcription but does so by recognizing two distinct operator sites (13).

Initial work in *E. coli* has showed that the Zur protein (EcZur) responds to zinc with an incredibly high affinity suggesting there is little free zinc within the cell (21). Subsequent characterization of the EcZur zinc binding sites suggested the protein has two independent zinc binding sites per monomer (22). The first site is thought to be analogous to the zinc structural site of the *E. coli* Fur protein (EcFur) and consists of conserved cysteine residues creating a binding pocket for zinc which is resistant to removal by EDTA (2). A cross species analysis of Fur family members shows variable conservation of this $ZnCys_4$ structural motif. For example, *Pseudomonas areginosa* Fur only contains one of the four conserved cysteine residues and therefore it is not surprising that the structural site is not observed in the crystal structure (23). However in PerR of *B. subtilis*, and Fur of *Heliobacter pylori* all four cysteines are conserved and form a stable structural zinc site needed for the dimerization of the protein (16, 27). Interestingly, the recent crystal structure of Nur, a nickel responsive Fur family member, from *Streptomyces coelicolor* provides another possibility. In this protein, the two CxxC motifs are conserved and form a $Cys_4$ site. However, mutation of any of the cysteines had no effect on protein dimerization or DNA binding activity, thus suggesting that although conserved, the cysteines are not essential in stabilizing protein folding (3).

The second EcZur zinc binding site was determined by EXAFS analysis to be a tetrahedral site comprised of a $S(N/O)_3$ environment (22). While no crystal structure of either the EcFur or EcZur has been completed there currently are five metallated crystal structures of Fur family members: Fur from *Psuedomonas aeruginosa* (PaFur) (23), Fur from *Vibrio cholerae* (VcFur) (24), FurB from *Mycobacterium tuberculosis* (MtZur) (19), Nur from *Streptomyces coelicolor* (ScNur) (3), and PerR from *Bacillus subtilis* (BsPerR) (14). The combined knowledge gained from these structures is that Fur family members have at least two and as many as three conserved metal binding

motifs. It was originally proposed based on the PaFur structure that site 1 served as the metal sensing site and site 2 served as a structural zinc site (23). However recent dynamic modeling evidence supports the hypothesis that site 2 serves as the metal sensing site (1, 17), site 1 plays a now yet unidentified role and a Zn-$Cys_4$ zinc site, not found in the PaFur protein, plays a structural role.

In light of the great diversity exhibited by Fur family members in their usage of these three conserved metal binding motifs to sense and respond to metal limitation, we have endeavored to understand how BsZur senses zinc levels at a biochemical level. Towards this goal we have created a suite of site mutants in each of the conserved metal binding motifs and tested their functions both *in vitro* and *in vivo*. From this work we conclude that BsZur contains a Zn-$Cys_4$ structural motif which is required for a functional protein. Additionally we provide evidence which challenges the assumption that site 2 functions as the sensing site and instead propose that in BsZur site 1 and 2 work cooperatively in zinc sensing.

### 3. Materials and Methods

**Bacterial Strains and Growth.** All strains are derivatives of the wild-type CU1065 (trp *attSPβ* ). Strain HB8010 (CU1065SPβ *yciC'-cat-lacZ*) was used for *yciC* promoter activity as previously described (9). *B. subtilis* was grown in LB media. Erythromycin (1μg/ml), lincomycin (25μg/ml), spectinomycin (100μg/ml), kanamycin (10μg/ml), neomycin (10μg/ml), and chloramphenicol (5μg/ml) were used for the selection of various *B. subtilis* strains. Zur mutants were created according to the Quick-Change Mutagenesis protocol published by Stratagene. A plasmid containing the *zur* gene fused to the FLAG sequence was used. The double mutants were created using Quick-Change mutagenesis using the plasmids gained from the single mutants as the template. Mutagenic PCR reactions were digested with *Dpn*I before transformation

into *E. coli* DH5α. Primers were synthesized by IDT, deoxynucleotides purchased from Perkin-Elmer, and reaction buffer and *Pfu* Turbo purchased from Stratagene. Plasmids were extracted from the *E. coli* DH5α transformations and transformed into a *zur* knockout strain of *B. subtilis* which also contained a Zur promoter reporter fusion.

**β-Galactosidase Assays**. 5 ml LB was inoculated (1:100) with overnight cultures. The cells were harvested when they reached mid log phase ($A_{600}$ of ≈0.4) and the β-galactosidase assay was performed by Miller's method as previously described (20) except that cells were lysed by the addition of lysozyme to a final concentration of 20μg/ml followed by a 30 min incubation of 37$^{o}$C. All assays were performed in triplicate and the values were averaged.

**Western Blot Analysis of Zur.** 5 ml LB media were inoculated (1:100 dilution) with strains grown in LB overnight. Cells were grown late log phase ($A_{600}$ of ~0.8) and harvested by centrifugation. The cell pellet was resuspended in 0.5 ml of Buffer A (20 mM Tris ·HCl (pH 8.0) 1mM DTT) and lysed by sonication. Cell debris were removed by centrifugation and the resulting supernatant was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and boiled for 10 minutes prior to being resolved by 12% SDS-PAGE. The proteins were transferred to a membrane (PVDF Millipore Immobion-P #IPVH 000 n10) at 100V for 40 minutes. The membrane was then blocked with blocking solution (Dry Milk dissolved in 20 mM Tris, 150 mM NaCl, .05% Tween-20) overnight. The membrane was incubated with polyclonal FLAG Antibody for one hour, washed with TTBS (20 mM Tris, 150 mM NaCl, .05% Tween-20), and incubated with anti-rabbit antibody conjugated to alkaline phosphatase antibody for 45 minutes. The membrane was then developed with 5ml AP buffer (100mM Tris ( pH 9.5), 100 mM NaCl, 5mM $MgCl_2$) and 1:100 dilution of NBT and BCIP. The Zur-FLAG protein has a molecular weight

of ~17 kDa as determined by ExPASy ProtParam tool and in agreement with the observed mobility on the SDS page gel.

**Purification of Zur, Zur-FLAG site mutants.** The *zur* open reading frame was amplified by polymerase chain reaction (PCR) using the forward primer (224) 5'-ATACATATGAACGTCCAAGAAGCG-3' and the reverse primer (220) 5'-ATAGGATCCTGTATTCACGAACGAAAATCG-3', creating *Nde*I and *BamH*I sites respectively (underlined). This PCR fragment was then cloned into vector pET-11a creating a T7 controlled *zur* gene (26). This plasmid was introduced into *E. coli* strain BL21(DE3 pLysS) with selection for $Cm^R$ (35 μg $ml^{-1}$) and $Amp^R$ (100 μg $ml^{-1}$). Glucose was added to the growth medium (0.5% w/v) to reduce the basal level expression of T7 RNAP. *E. coli* cells from 5 ml of overnight culture in LB medium were inoculated into 1 liter of fresh LB medium containing 0.5% glucose, 34 mg/liter chloramphenicol, and 100 mg/liter ampicillin. Isopropyl 1-thio-β-D-galactopyranoside (1 mM final concentration) and zinc (50 μM final concentration) were added at $A_{600}$ of ~0.4, and the cells were allowed to grow for an additional 4 h at 30°C. Cells were harvested by centrifugation, and the cell pellets were resuspended in 10 ml of buffer A (20 mM Tris·HCl (pH 8.0), 100 mM NaCl, and 5% (v/v) glycerol) containing 20 mM EDTA. After lysis by sonication, cell debris were removed by centrifugation at 10,000 rpm for 5 minutes at 4°C, and 3 ml of the resulting supernatant was loaded onto a heparin column. Bound proteins were eluted with a linear gradient of NaCl. Fractions containing Zur were determined by a 12% SDS-PAGE gel and the chosen fractions were applied to a desalting column followed by a Mono Q column all using an FPLC system (GE Healthcare). The Zur-containing fractions were further purified on a Superdex 200 HiLoad 16/60 column (GE Healthcare) equilibrated with Chelex-100-treated buffer A without EDTA. The fractions containing Zur were pooled and kept at -80°C.

**Determination of Protein Concentration.** The concentration of purified Zur was determined by measuring $A_{277\ nm}$ using the calculated value of $\varepsilon_{277\ nm} = 13{,}785\ M^{-1}\ cm^{-1}$. The concentration of proteins in the cell crude extract was determined by Bio-Rad protein assay using bovine serum albumin as a standard.

**Electrophoretic Mobility Shift Assay (EMSA) of Zur Binding**. PCR fragments containing the *rpmGC* promoter region and a promoter region not known to bind Zur were generated by PCR, end labeled with $\alpha$-$^{32}$P by polynucleotide kinase (Epicenter), purified using NucAway columns (Ambion) according to the manufacturer's instruction, and used in EMSA experiments as previously described (9).

**Determination of Percent Activity of Purified Zur-FLAG**. The PCR product of the *ytiA* promoter region containing the Zur operator sequence (Zur box) was quantified using a Nano Drop machine (ND-1000). An EMSA was performed (as described above) with a $\alpha$-$^{32}$P labeled *ytiA* fragment. Known quantities of the same fragment were added as cold DNA with increasing amounts of Zur protein. The percent activity calculation assumes that one dimer of Zur is sufficient to bind and fully shift one operator containing fragment of DNA.

**PAR Assay.** Purified Zur proteins were analyzed for zinc content by monitoring absorbance at 494nM in a Lamda 25 Spectrometer (Perkin Elmer) of the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR). Under our experimental buffer conditions (PAR buffer: 40mM Tris pH 8.0, 5% glycerol, .5% SDS) the absorption maximum of the $Zn^{2+}$-PAR complex was observed at 494 nM and had a linear relationship to zinc concentrations from 0-8μM. Known amounts of purified proteins were added to PAR buffer conditions along with 0.1mM of PAR and 100μM of $H_2O_2$ and incubated at room temperature for 15 minutes. After oxidation by $H_2O_2$, the absorbance at 494nM was read and the total amount of released zinc calculated.

**PAR-PCMB Assay**. Zur proteins were analyzed for their zinc content by the reversible thiol-mercaptide bond forming compound p-chloromercuribenzoic acid (PCMB). Assay was performed as previously described (15).

**Zur Operator Protection Assay.** The following primer sets: (gcggtacatagccgatgcgc, ggcggataatgctgcgaaaagaagc) and (cggttggagtctttactgat, ttttcatctgttctggaaaggc) were used to PCR amplify the *znuC* promoter fragment from *E. coli* and the *yciC* promoter fragment from *B. subtilis*. In addition to containing their native Zur box both promoters also contain a native restriction site internal to the Zur operator sequence (Psi1 for EC and DraI for BS). Promoter fragments were end labeled as described above. 1μM of Zur protein, 1mM DTT, 200μM TPEN or EGTA with increasing amounts of Zn(II) were combined and allowed to equilibrate at room temperature for 10 minutes. Free zinc levels were calculated using the MaxChelator program: (http://www.stanford.edu/~cpatton/maxc.html). The labeled DNA is then added and allowed to incubate for 10mins followed by addition of 2 units of enzyme and incubation at 37$^{o}$C for 30 minutes. The reaction is stopped by the addition of 2μL of 10X Stop Solution (5% SDS, 0.025% bromophenol blue, 10mM EDTA) and heat inactivation at 98$^{o}$C for 10 minutes. Samples were run on a 8% Native gel at 90V for 60 minutes and visualized as described above.

## 4. Results and Discussion

**Zinc homeostasis as a function of externally added zinc**. Bacteria have an extraordinary ability to acquire zinc from their environment (12). To determine at what level of externally added zinc uptake and efflux responses are initiated in *B. subtilis*, we monitored the promoter activity of both *yciC* (Zur controlled-zinc uptake/mobilization) and *cadA* (CzrA controlled-zinc efflux) in minimal media with varying levels of added zinc. The Zur controlled promoter showed high activity until

approximately 1μM of zinc (Fig. 2.1 open rectangles). Mirroring that response, the CzrA controlled promoter did not show activity until 10μM added zinc and full activity was observed by 80μM zinc (Fig. 2.1 closed diamonds). Previous work in determining total zinc concentrations for *E. coli* grown in minimal media suggested that zinc concentrations easily reach 200μM inside the cell, 2000X greater than the zinc concentration of the media (21). While we cannot estimate the percent of zinc which is taken up by *B. subtilis* or its efficiency in concentrating zinc intracellularly, our values appear to be in a similar range when compared to the total zinc quota determined for *E. coli* (21).

**Zur's zinc binding affinity *in vitro*.** Having determined at what concentrations of external zinc Zur repressed *yciC* expression, we next monitored *in vitro* what zinc concentrations Zur could sense and thus resulting in binding to and protecting of its operator sequence from restriction digest (Fig. 2.2). As an initial control for the validity of our assay, we purified EcZur and tested its binding affinity for zinc. Our results demonstrate that EcZur has a femtomolar affinity for zinc which is in agreement with its published characterization as expected (Fig. 2.2 open triangles and (21). We then proceeded to measure the level of free zinc at which the BsZur protein is able to bind to its operator. Our results indicate that BsZur binds zinc with a comparable affinity to that of EcZur (Fig 2.2 filled circles). This implies that as in *E. coli* there exists little to no free zinc within the *B. subtilis* cytosol (21).

**BsZur possesses three proposed metal binding sites.** To identify candidate residues responsible for sensing zinc or involvement in forming a structural zinc site in BsZur, we took three complementary approaches: First we performed homology modeling to create a hypothetical structure of BsZur with three of the published crystal structures (BsPerR, PaFur, and MtZur). In each case the resulting structure resulted in comparable binding site assignments of conserved residues and therefore the PaFur

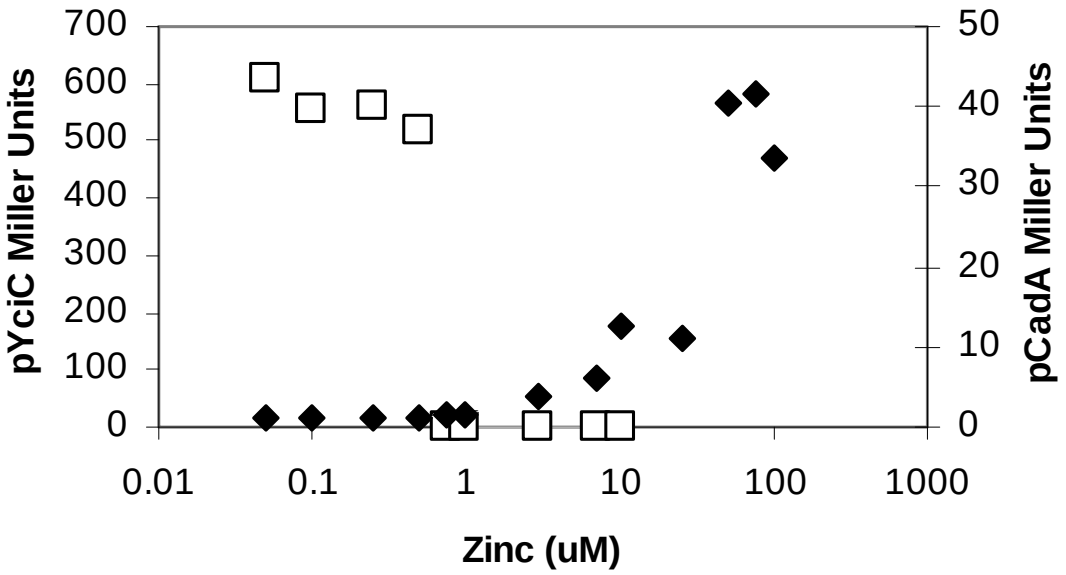



Figure 2.1 Zinc uptake and efflux as a function of externally provided zinc. Promoter fusions of a CzrA controlled zinc efflux ATPase, *cadA* (closed diamonds) and a Zur controlled proposed metallochaperone, *yciC* (open squares) grown in ZSMM with added zinc as noted.

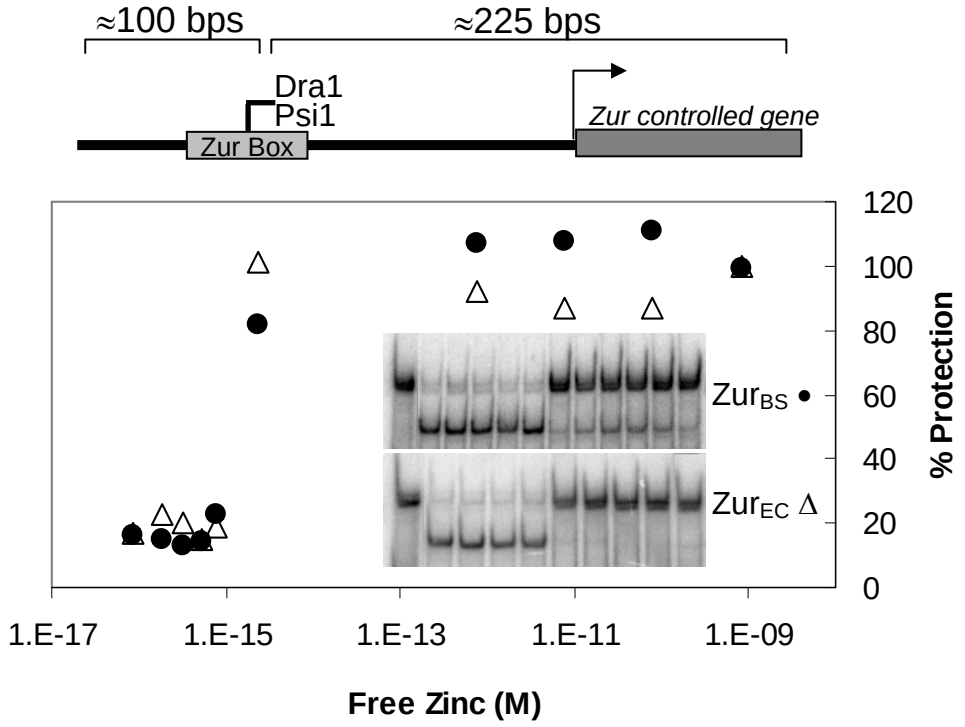



Figure 2.2 Zinc binding affinity of Zur proteins from *Bacillus subtilis* (ZurBS closed circles) and *Escherichia coli* (ZurEC open triangles). Promoters which contained a known Zur operator site and a native restriction site were labeled and incubated with Zur, restriction enzyme and increasing amounts of free zinc. Zur's affinity for Zn is monitored by its ability to bind the promoter fragment and protect it from digestion. Percent protection was calculated by a ratio of band intensity values as determined by the program ImageQuant data analysis software.

modeled structure is shown for reference (Fig 2.3A). Second, we aligned nine Zur protein sequences from a diversity of bacterial species to search for conservation of important residues (Fig 2.3B). Finally, we aligned the BsZur protein with all of the published metallated Fur family regulators (Fig 2.3C). All of the site mutants created based on these approaches are highlighted in green on the BsZur protein sequence. (Fig 2.3C).

On first inspection of the homology modeled BsZur protein (Fig 2.3A), the three previously observed metal binding sites of other Fur family members became readily apparent. The $Cys_4$ structural site (red residues Fig 2.3A) appears to stabilize a beta sheet interaction which would affect the localization and stability of the dimerization domain. In our protein this pair of conserved CxxC motifs consists of residues C95, C98, C132 and C135.

As a first approximation of the proposed sensing site, site 2 residues were defined as E70, C84, H90 and H92 (blue residues Fig 2.3A). The alignment of Zur proteins from other species shows high conservation of these four residues alluding to their importance in protein function (Fig 2.3B). Additionally, when aligned with the only crystal structure of a Zur protein, MtZur, these residues are well conserved (FurB-D62, C76, H81 and H83) (Fig 2.3C). Since zinc prefers a tetra-coordinate binding pocket and many of the crystal structures are for regulators which bind iron, needing five ligands, we expected one of the residues not to be conserved in the site 2 binding pocket of Zur. Aligning the protein sequences shows that BsZur lacks conservation at the comparable residues of H37 in BsPerR (Y34 in BsZur – Fig 2.3C).

Site 1 was visualized in three of the five crystal structures PaFur, VcFur and MtZur (19, 23, 24). While the function of this site remains unknown, the four residues that compose this site in the three crystals are highly conserved and in only one instance is there a variant amino acid. Additionally, when site 1 from the three crystal

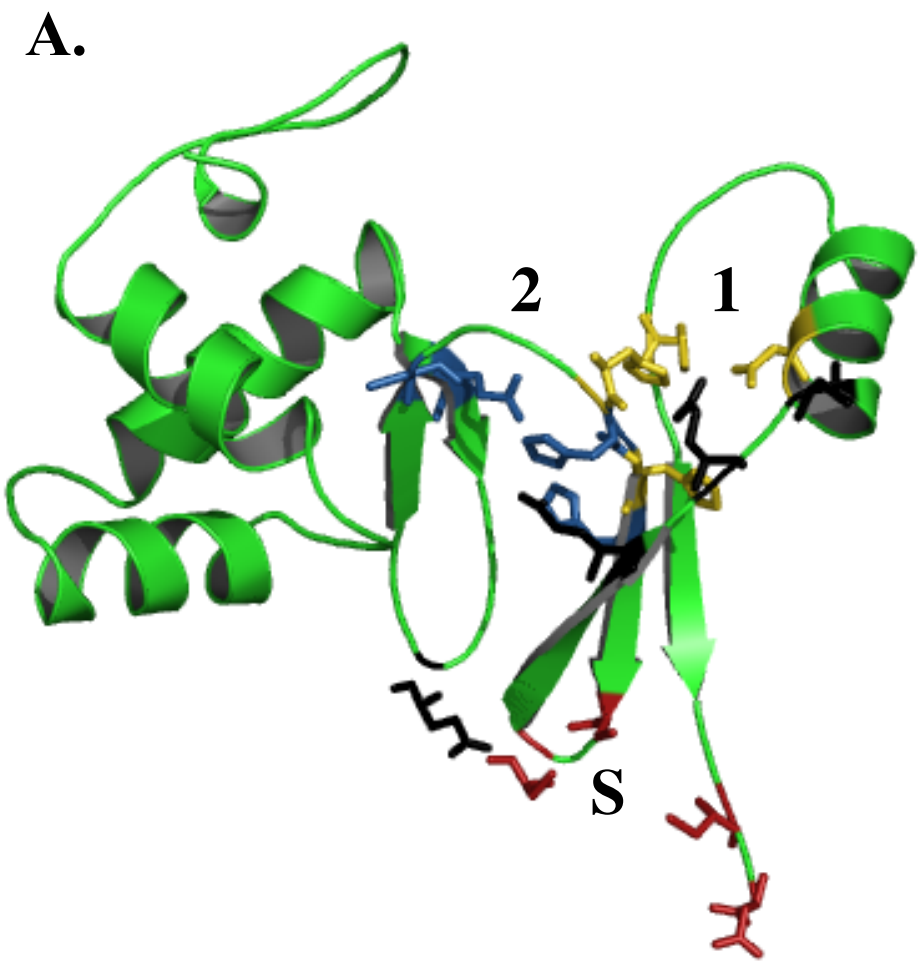
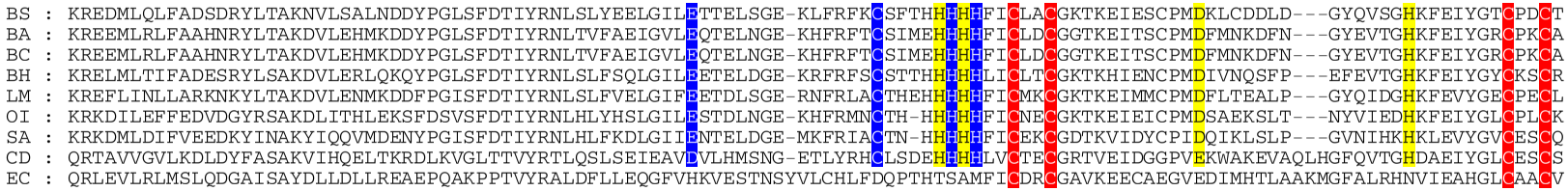



Figure 2.3A - A monomer of BsZur was modeled on *Pseudomonas aurginosa* Fur using SwissModel. Residues making up each of the proposed metal binding motifs are shown in red (structural), blue (site 2), yellow (site 1) and black (other conserved residues).

**B.**




**C.**


Figure 2.3 cont. B. ClustalW alignment of Zur proteins (*Bacillus subtilis* (BS), *Bacillus anthracis* (BA), *Bacillus cereus* (BC), *Bacillus halodurans* (BH), *Listeria monocytogenes* (LM), *Oceanobacillus. iheyensis* (OI), *Staphylococcus aureus* (SA), *Corynebacterium diphtheriae* (CD), *Escherichia. coli* (EC)) illustrating the conservation of the three metal binding motifs. C. ClustalW alignment of five Fur family published crystal structures: *Streptomyces coelicor* Nur (SC.Nur), *Vibrio cholerae* Fur (VC.Fur), *Mycobacterium tuberculosis* FurB (MT.FurB), *Pseudomonas aeruginosa* Fur (PA.Fur), *B. subtilis* PerR (BS.PerR). Residues observed to bind metals are shaded as outlined in (A) Shaded residues in BS.Zur are those which were targeted for site-directed mutagenesis in this study. Note alignments are truncated at C and N terminal of protein to highlight region of interest.

structures are compared they are virtually super imposable. In the BsZur sequence three of the four residues are conserved, H89, H91, and H124. E101 from MtZur is not conserved in BsZur however, it is likely both from the homology model and the conservation observed in other Zur proteins that D110 serves as the fourth ligand in site 1 for BsZur.

Finally, residues which showed high conservation in the comparative Zur sequence alignment (Fig. 2.2B – residues E77, E103, C107, E127) or are known good zinc ligands (E105 and C113) were also chosen for mutagenesis. We predict that with the observed diversity in metal binding sites of Fur family members it is possible a new variation exists for sensing and binding zinc in *B. subtilis*. These residues are labeled "others" in Fig 2.4 and colored in black in Fig 2.3A.

**A Zn-Cys$_4$ is required for protein function.** To test the role of the two conserved CxxC motifs in BsZur function, each cysteine was mutated to a serine and the mutant Zur protein was tested for its ability to repress a Zur regulated promoter fusion *in vivo*. Mutation of any of the four cysteines causes a severe derepression similar in degree to that observed in a *zur* null strain (Figure 2.4). This is consistent with previous data from the characterization of BsPerR (16) that this motif serves as a structural site required for proper protein folding in BsZur. To test this hypothesis, we performed a Western-blot analysis on each of the structural site mutants (Suppl. Fig 2.1). In every case mutating a structural site residue resulted in a reduced mobility, representing a protein with an unstable structural site, which unlike WT BsZur Zn-Cys$_4$, is no longer resistant to SDS treatment and subsequent boiling (16). It is interesting to note that the level of derepression observed decreases for C132 and C135 mutants. It is possible these mutations still are able form a sub-optimal structural site producing a small fraction of active Zur protein which enables a limited amount of repression *in vivo.*

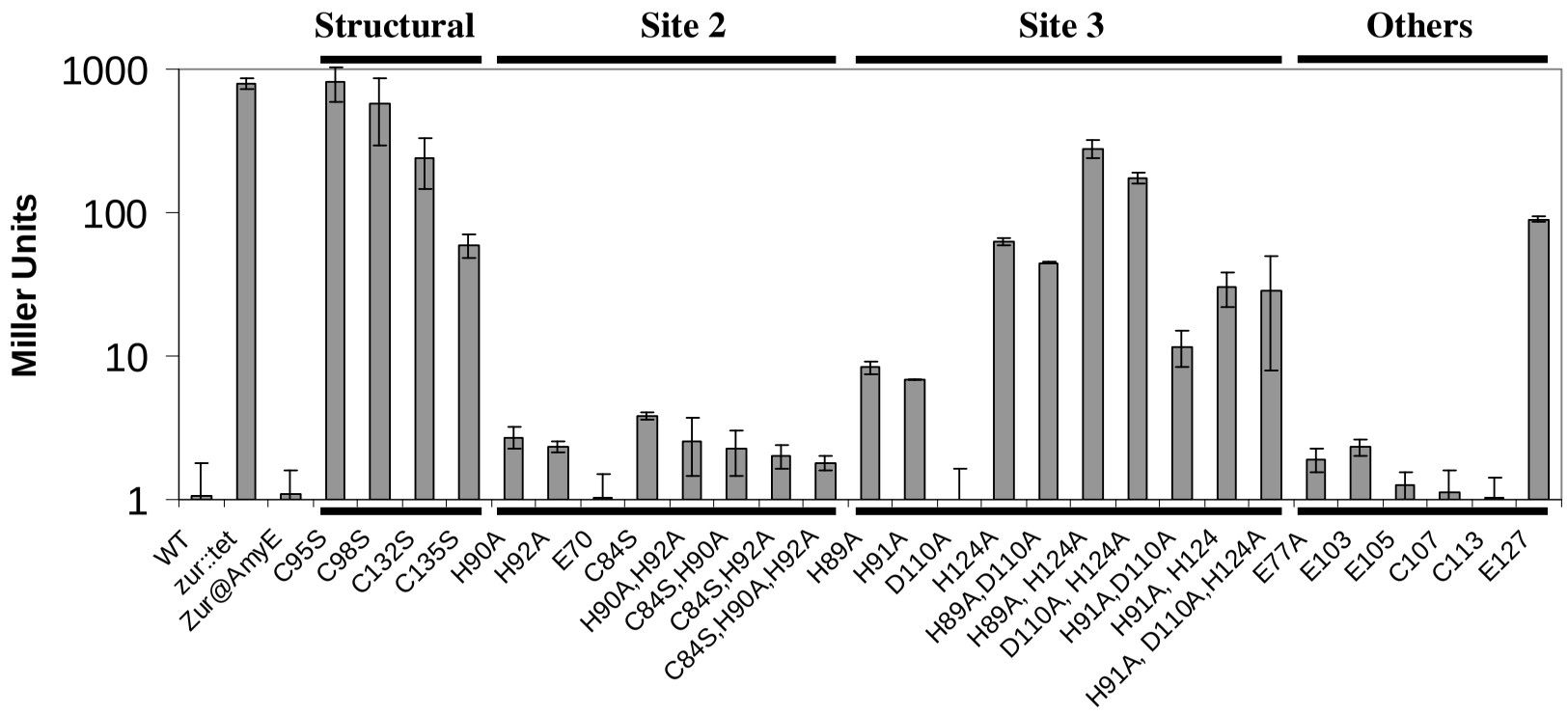



Figure 2.4 *In vivo* derepression of the suite of BsZur site mutants. Structural mutants show full depression, in comparison site 1 mutants have a lesser but still strong derepression and finally even a triple site 2 mutant shows only modest derepression. All mutants are made from a plasmid which integrates at the ectopic locus *amyE* and contains the sequence for a Zur-flag tagged protein. Mutants are grouped above according to their suspected metal binding site location: structural Zn-Cys4 site, site 2 (proposed regulatory site), site 1 (unknown function) and other residues which showed sequence conservation.

However due to the instability of this site, SDS and boiling removes all traces of an intact structural zinc site when visualized by a Western Blot.

**Neither Site 1 nor Site 2 disruption abolishes Zur repression *in vivo*.** To determine which of the remaining two metal sites functions as the regulatory zinc binding site, single double and triple mutants of the proposed site 1 and 2 residues were created and tested for repressor activity and stability *in vivo* (Fig. 2.4 and data not shown—all site 1 and 2 mutants show WT mobility by Western Blot analysis). Surprisingly, even a triple site 2 mutant (C84, H90, H92) only showed a very modest derepression *in vivo* (Figure 2.4). This result can be explained in several ways. First, it is possible that BsZur uses alternate amino acids to create its regulatory site. While this seems unlikely given the conservation of the residues, it is not without precedent. In the recently published structure of ScNur, the $Ni^{+2}$ binding site is not like any other metal binding site currently characterized in Fur family members (3). At the onset we chose other zinc binding residue candidates to increase our chances of identifying all ligands of the zinc binding pockets. Examining that list there are three possible residues which according to our homology modeled structure could localize to site 2 (E77, E103 and E127). Of these three residues only E127 shows derepression *in vivo*. Alternately, it is possible that the chosen residues in fact do comprise the regulatory site and that due to weakening of the protein's zinc sensing ability a low level of expression of the Zur regulon is allowed. This slight induction could hypothetically increase the intracellular levels of zinc and thus potentially overcome the decrease in zinc binding affinity of the site 2 mutants. This cascade of events would lead to what appears to be near WT repression levels. However, even if no de-repression of Zur occurs and intracellular zinc levels remain constant, it has been reported that a triple site 2 mutant in *B. japonicum* Fur still showed WT binding of a *fur* promoter *in vitro*

and thus may provide an alternate explanation to this *in vivo* result (8). Finally, it is also possible that site 1 is in fact the regulatory site or acts cooperatively to site 2.

While site 1 was initially labeled as the regulatory metal binding site for PaFur (23), this is no longer thought to be the case. In the structures where a metal is bound at site 1, it can be easily removed upon dialysis with EDTA. Somewhat surprisingly, mutation of site 1 residues in BsZur caused a moderate derepression phenotype *in vivo* (Fig. 2.4). Clearly the most critical residue in this site is H124. However mutating H89 or H91 cause a more significant phenotype than any of the site 2 residues combined. Additionally in the case of H89, a double mutant phenotype is greater when combined with either H124 or D110. Interestingly, a D110 single mutant has no phenotype except when combined with other site 1 residues suggesting that these residues are working cooperatively to create a binding site. These data lead to two likely scenarios. First, given its proximity to the dimerization helix it is possible site 1 serves as a secondary structural zinc site. Disruption of this site causes inefficient dimer formation resulting in derepression even though metal sensing at site 2 remains intact. Alternately site 1 could be the "bona-fide" zinc sensing site or at least a back-up sensing site to site 2. It was our expectation that if we were to abolish zinc sensing through our mutagenesis the Zur protein would become "zinc-blind" and Zur regulated promoters would be fully derepressed in all conditions. While mutations in neither site achieved this anticipated goal, clearly some of the double site 1 mutants approach this level of derepression (Fig 2.4 -H89,H124 and D110, H124). Since all of these mutants are stable and expressed at WT levels *in vivo* (data not shown), the observed derepression is presumed to be a defect in zinc binding.

**Both site 1 and 2 mutants are still able to bind zinc.** To determine if the depression observed in site 1 and 2 mutants is caused by an altered ability to bind zinc, two independent zinc binding assays were preformed *in vitro*. First Zur WT and

mutant proteins were purified in the presence of 10mM EDTA to remove any loosely associated metals. Before storage, pure proteins were run on a sizing column equilibrated with chelexed buffer A to remove the EDTA. These proteins were then tested for zinc content by a PAR and PCMB assay. Both of these assays use the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) to monitor zinc release by a change in absorbance at 494nm. In the PAR assay the protein is disrupted by the addition of high levels of $H_2O_2$ where in contrast in the PCMB assay PCMB forms mercaptide bonds with thiol groups thereby releasing any cysteine bound zinc in a reaction that is reversible with the addition of reductant.

For the ZurWT protein both assays determined that the purified protein contained approximately .7 zinc atoms/monomer (uncharged column Table 2.1). This number is within the expected range of 1 zinc/monomer due to the presence of the highly stable and EDTA resistant Zn-$Cys_4$ site (16). When the WT protein was subsequently incubated with zinc, the zinc content of the protein changed by 1 zinc/monomer confirming that BsZur like other Fur family members binds a maximum of 2 zinc atoms/monomer (Table 2.1). It should be noted that site 1 does not contain any cysteine residues to which PCMB can bind and release zinc. However, it is not clear if upon removing the structural zinc from the $Cys_4$ site (and possibly from site 2 residue C84) whether site 1 remains in a conformation competent to bind zinc.

Both site 1 and site 2 mutants showed a lower occupancy of the $Cys_4$ structural site (Table 2.1). This could be due in part to the proximity of the C-terminal FLAG tag to the structural site. Having this epitope creates a bulky C-terminal which may reduce some of the EDTA resistance of the WT structural site. Two interpretations can follow from these data. First one could assume that the active portion of the protein is equal to those which contain a structural zinc atom and therefore upon incubation with zinc this portion should double. Alternately, the structural site could still be correctly

Table 2.1 – Zinc content of Zur proteins

| | PAR assay | | PCMB Assay | |
|---|---|---|---|---|
| | Uncharged | 25 mol equiv | Uncharged | 2 mol equiv |
| Zur(wt) | 0.7 | 1.75 | 0.67 | 1.63 |
| Zur(Flag) | 0.64 | 1.58 | 0.39 | 0.86 |
| Zur(Flag)-H124A | 0.39 | 1.49 | 0.35 | 0.93 |
| Zur(Flag)-C844A | 0.45 | 1.3 | 0.39 | 0.88 |

All values shown in table are in units of zinc atoms/monomer of Zur

folded and competent to bind zinc and not EDTA resistant. Thus upon incubation with zinc, some portion is diverted to the unoccupied structural site and another portion is bound by the sensing site. Regardless of how the data is interpreted, what is clear is that both site 1 and site 2 mutants are not abrogated in their ability to bind a zinc atom in addition to the structural zinc. This could be due to the sites playing redundant roles in zinc sensing or the amount of added zinc was in excess of the hypothetical zinc binding affinity defect.

**Site 1 mutants show altered DNA binding activity.** To further clarify the observed *in vivo* derepression phenotype (Fig. 2.4) and the *in vitro* zinc content studies of the site 1 mutants (Table 2.1), two DNA binding assays were performed. BsZur has been previously shown to require zinc binding to associate with its operator sequence (9). We have used a native restriction site within the Zur operator sequence of the *yciC* promoter to assess Zur binding as a function of free zinc (Fig 2.2). Using this same assay with the mutant Zur proteins we can assess if these proteins have a detectable zinc binding defect. As shown in Fig 2.5A, the Zur-FLAG WT protein has a comparable affinity for zinc as the untagged Zur. However, both of the tested site 1 mutants display a grossly altered ability to bind zinc. We reason that this observed phenotype is due to a zinc binding defect and not a direct DNA binding defect because added zinc should not be able to reverse a specific DNA binding mutation. This result is surprising since when zinc content of the same mutant proteins were measured, all tested mutants showed the ability to bind a total of two zinc atoms per monomer. It is possible that in this assay with significantly lower free zinc levels that the zinc binding defect of these proteins are revealed.

To further confirm this finding, EMSA analysis was performed on the same selected proteins (Fig. 2.5B). Again the Zur-Flag WT protein has a similar $K_d$ when compared the published values of the untagged proteins (10, 11). Consistent with the

protection assay, a binding defect is observed in both of the site 1 mutant proteins. Specifically for the H124A mutant and the H124A, H89A double mutant the estimated $K_d$ values decreased 6 and 9 fold respectively when compared to the Zur-Flag WT protein (Fig. 2.5B).

**Some site 1,2 double mutants show full derepression.** Based solely on our DNA binding and protection experiments (Fig. 2.5), we would conclude that the mutants have a zinc binding defect. However, our zinc content data provides a conflicting view and suggests it is possible the two sites play a redundant role in zinc sensing (Table 2.1). To help differentiate between these two possibilities, we constructed strains mutated in both site 1 and site 2. We reasoned if the sites were acting redundantly disrupting both sites would cause a full derepression phenotype due to the protein becoming "zinc-blind". However, if the two sites were contributing to Zur's function independently a partially active protein may still be obtained. The data obtained from these experiments however raises a third intermediary possibility. When H124 (site 1) is combined with any of the three suspected site 2 residues which showed derepression *in vivo* when mutated (H90, H92, C84), the protein is no longer stable and therefore full derepression is observed (Fig. 2.6). However when three of the four suspected site 1 residues are mutated (H89, H91, H124) the protein is stable and only a modest derepression is observed. Additionally, when other combinations of site 1, 2 mutants are created that do not include the H124 residue derepression is fairly modest (H90, H92, D110 or C84, D110). Moreover, if E127 is an authentic site 2 residue then two more site 1,2 mutants which lack H124 also do not show full derepression (D110, E127 and H89, H91, E127). The fact that these site 1,2 mutants containing H124 show a reduced mobility upon Western analysis implicates $Cys_4$ structural site instability. It is plausible that H124 given its proximity to C132 and C135 might affect correct folding of the structural zinc site. It is also possible that

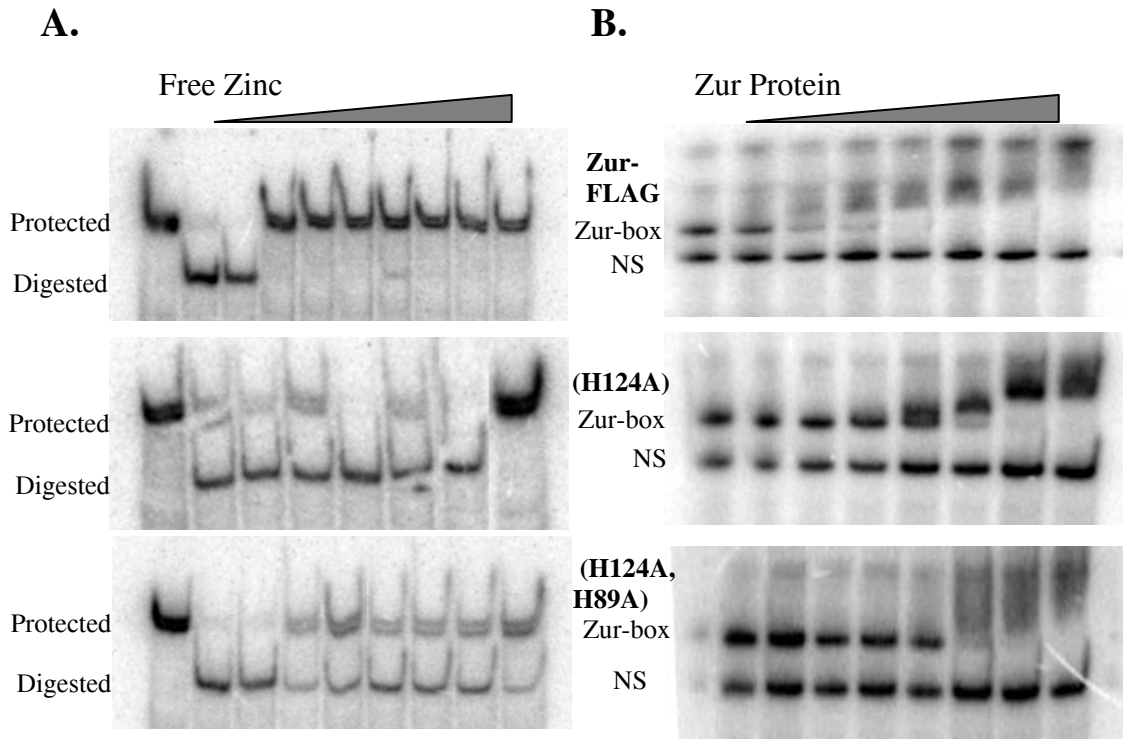



Figure 2.5 Site 1 mutants show a decreased binding affinity for zinc and DNA. A. Mutation of H124 of site one causes a significant reduction in zinc affinity when measured in a protection assay. Approximate affinities as determined by this assay were WT-$10^{-15}$, H124A-$10^{-10}$ and H124A,H89A-$10^{-8}$ B. This reduction in zinc binding presumably translates into the observed reduction of DNA binding affinity as measured in this EMSA. Bs Zur shifts only its operator containing fragment (Zur-box) and not the non specific promoter control (NS). Approximate $K_d$'s were determined for the three Zur protein variants (WT-3.5nM, H124A-20nM, H124A,H89A-30nM).

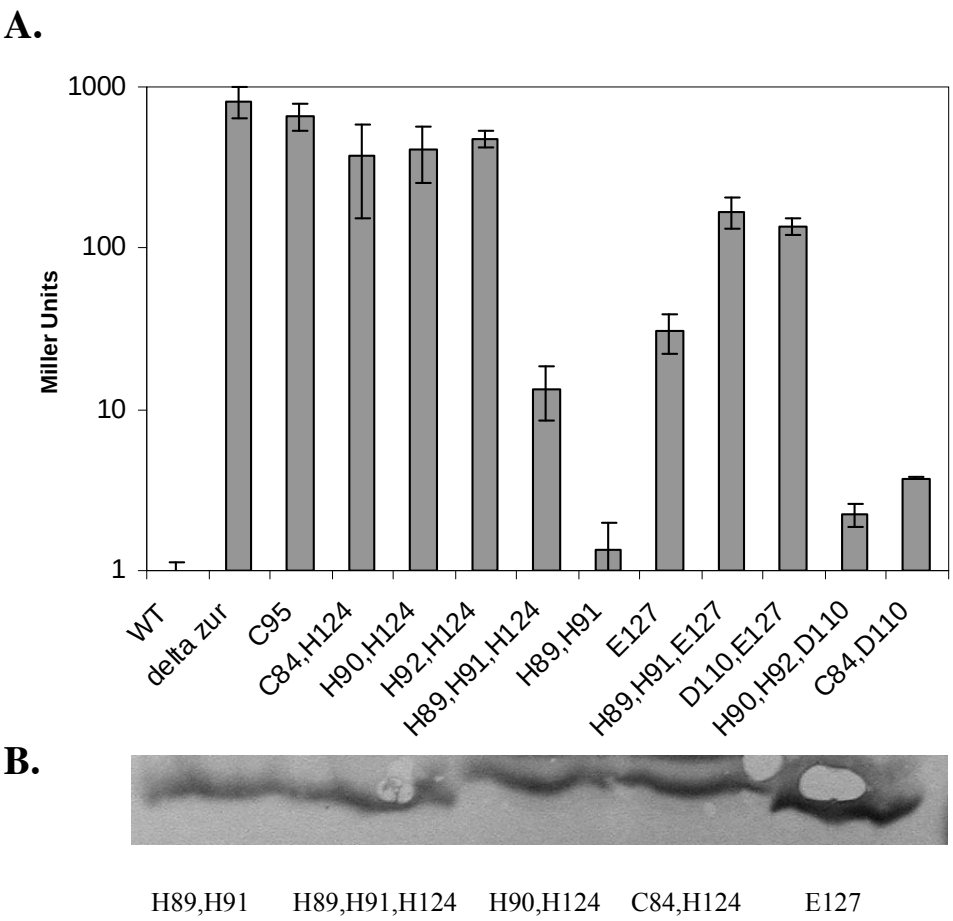



Figure 2.6 Some site 1,2 mutants for full derepression *in vivo* A. β-galactosidase experiments show some combined site 1 and 2 mutations result in full derepression. The H124 residue of Zur appears to be critical to Zur's function because when paired with any of the suspected site 2 residues (C84,H90,H92) full derepression is observed. However a triple site 1 mutant shows only a modest affect. B. Western analysis confirms that the H90, H124 and C84, H124 mutants are not stable in vivo.

site 1 itself plays a structural role for the protein. What remains to be clarified is why site 1 is destabilized only upon mutating residues suspected to be in site 2.

**5. Concluding Remarks**

We started this work with the goal of understanding the details of the biochemical basis of zinc sensing in *B. subtilis*. While many questions remain, we have confirmed several important biochemical details of Zur's function. For instance, we have confirmed that Zur has a very high sensitivity for zinc comparable to what was reported for its counterpart in *E. coli* (21). This provides insight to the relative free levels of zinc within the *B. subtilis* cytosol. Additionally, we know that the conserved CxxC motifs in the BsZur protein are essential for the protein to fold and function properly. Finally, we have identified several amino acids which appear to have critical roles in the proper functioning of Zur.

For each question this study has addressed several additional questions have also been raised. First, it remains unclear the role of site 2 of Zur. Several follow up studies should be done to address this concern. One of the proposed explanations for the lack of derepression phenotype in site 2 mutants was that a slight derepression of the Zur regulon results in a possible increase in intracellular zinc levels. If derepression was measured in a media condition and genetic background which induced zinc starvation this affect might be eliminated thus increasing the fold induction of site 2 mutants. If this held true, the precise contribution of each of the site 2 residues could be visualized. This has been done preliminarily for WT and site 1 mutants (see Suppl. Fig 2.2). For a Zur WT a slight derepression is observed under zinc starvation conditions. When site 1 mutants are measured in these same conditions their absolute level of activity increases dramatically but the fold change as compared to WT is similar to rich media conditions. If site 1 acts as a secondary structural site

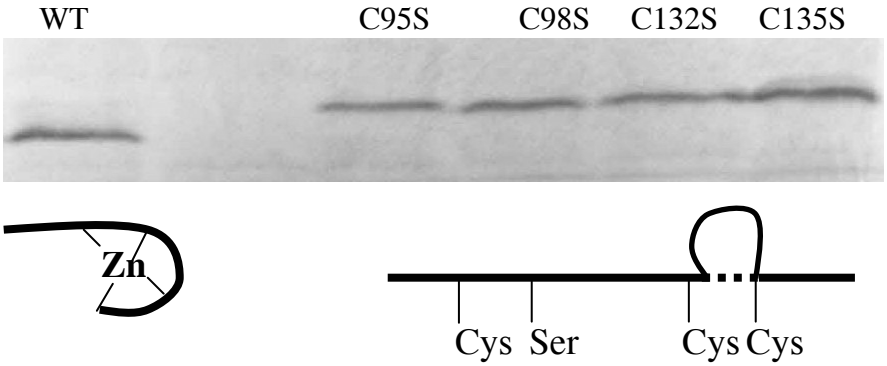
we would expect similar fold induction for each condition. It would be informative to test the site 2 mutants in these conditions and compare their fold induction.

A second study which would also be helpful in determining the role of site 2 would be to repeat the zinc content studies in non FLAG tagged Zur proteins. Due to the inconsistency of the data obtained from the zinc content and DNA binding assays in determining zinc occupancy of the proteins, a true understanding of the mutant protein's ability to bind zinc was not achieved. One possible explanation as to why the mutant proteins were able to bind zinc would be due to zinc binding to the FLAG tag moiety. Five of the eight total amino acids of this epitope are aspartic acid residues, a known zinc ligand.

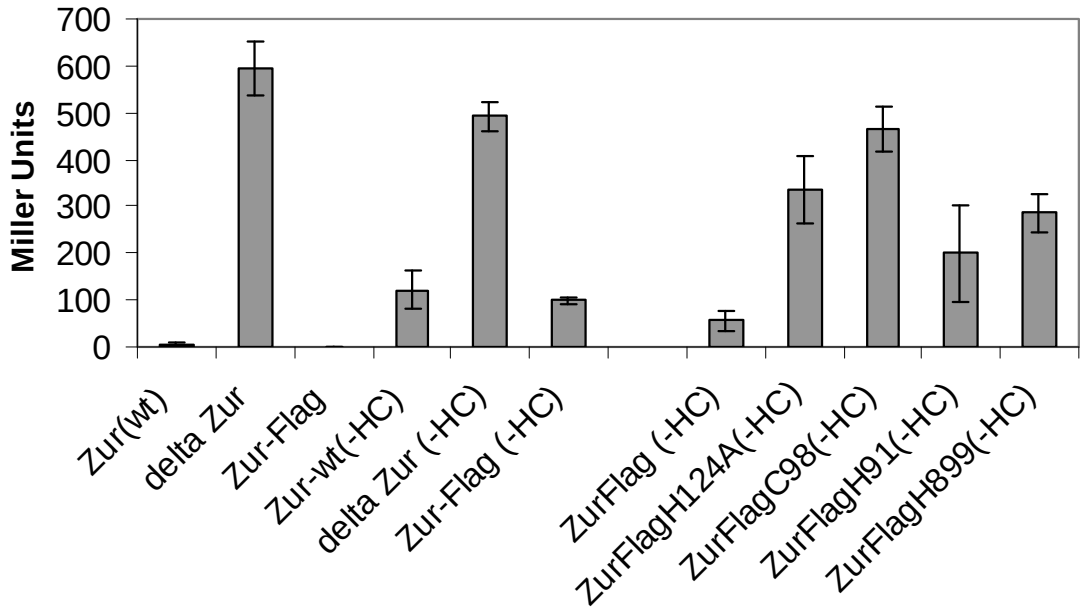
The second major question raised by this work is the role of site 1 and its possible interaction with site 2. A straightforward first step would be to repeat these studies in non-epitoped tagged proteins. If the C-terminal FLAG tag slightly destabilizes the C-terminal zinc structural site, additional instability caused by a mutation of site 1 located in close proximity to the structural site may result in a non-optimal repressor. This hypothesis is strengthened by the observation that the two greatest single mutation affects observed (not including structural site mutations) were H124 and E127.

All of the questions raised by this study would likely be significantly aided by a crystal structure of this protein. However clearly there is much more work to be done before we comprehensively understand the biochemical mechanisms of sensing metals by Fur family members.

APPENDIX




Supplemental Figure 2.1 Western analysis of structural site mutants. Mutation of any of the conserved cysteine residues creates a protein with higher mobility as observed on a SDS page gel. In WT proteins the zinc structural site is resistant to SDS and boiling and therefore remains folded as depicted above left. When even one of the four cysteines is mutated, this structure linerizes creating a protein with higher mobility. This phenotype has been described previously (see ref. 14) and produces a non-fucntional protein.




Supplemental Figure 2.2 BsZur site mutants in zinc starved conditions. Using zinc starvation conditions to observe functionality of BsZur site mutants. β-galactosidase measurements in ZSMM of a Zur regulated promoter fusion were performed on the following set of three: WT, Δzur, Δzur *zur*-FLAG-AmyE in two strain backgrounds (WT and Δ*ycdH* Δ*yciC* (-HC)). Zur wt shows modest derepression when grown in conditions which induce zinc starvation and site show an exaggerated derepression due to the zinc starved conditions.

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CHAPTER 3

# REGULATION OF THE *BACILLUS SUBTILIS YCIC* GENE AND INSIGHTS INTO THE DNA-BINDING SPECIFICITY OF THE ZINC-SENSING METALLOREGULATOR ZUR*

## 1. Summary

The *Bacillus subtilis* Zur protein regulates zinc homeostasis by repressing at least ten genes in response to zinc sufficiency. One of these genes, *yciC*, encodes an abundant protein postulated to function as a metallochaperone. Here, we used a genetic approach to identify the *cis*-acting elements and *trans*-acting factors contributing to the tight repression of *yciC*. Initial studies led to the identification of only *trans*-acting mutations and, when the selection was repeated using a transposon library, all recovered mutants contained insertionally inactivated *zur*. Using a *zur* merodiploid strain, we obtained two *cis*-acting mutations that contained large deletions in the *yciC* regulatory region. We demonstrate that the *yciC* regulatory region contains two functional Zur boxes: a primary site (C2) overlapping a $\sigma^A$ promoter ~200 bp upstream of *yciC*, and a second site near the translational start point (C1). Zur binds to both of these sites to mediate strong, zinc-dependent repression of *yciC*. Deletion studies indicate that either Zur box is sufficient for repression, although repression by Zur bound to C2 is more efficient. Binding studies demonstrate that both sites bind Zur with high affinity. Sequence alignment of these and previously described Zur boxes suggest that Zur recognizes a more extended operator than other Fur family members. We have used synthetic oligonucleotides to identify bases critical for DNA-

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binding by Zur. Unlike Fur and PerR, which bind efficiently to sequences containing a core 7-1-7 repeat element, Zur requires a 9-1-9 inverted repeat for high affinity binding.

## 2. Introduction

Zinc is an essential nutrient used both as a structural cofactor for protein folding and as a catalytic cofactor for many enzymes. However, at high levels, zinc can be toxic (3). It is therefore crucial that cells tightly regulate zinc levels within the cell. This involves, in part, the regulated expression of uptake and efflux proteins. It has been noted that the equilibrium levels of free zinc, as sensed by well characterized *Escherichia coli* zinc metalloregulators Zur and ZntR, are in the femtomolar range (25). In *Bacillus subtilis*, we have observed a similar high affinity for the homologous Zur protein (our unpublished results). Since this corresponds to essentially no free zinc in the cell, zinc is presumably chaperoned within the cell by one or more metallochaperones analogous to those that direct intracellular copper trafficking (9). To date, however, no zinc metallochaperone has been described in detail. One candidate for such a function is *B. subtilis* YciC, an abundant cytosolic protein regulated by Zur.

The *B. subtilis* Zur protein represses expression of at least ten genes in response to zinc sufficiency (14, 26). Orthologs of Zur have been found in a wide range of species including *E. coli, Mycobacterium tuberculosis, Salmonella enterica* and *Staphylococcus aureus* (4, 20, 21, 27). In *B. subtilis*, the genetic response to zinc starvation includes, as expected, the derepression of a high affinity zinc uptake system, an ABC transporter encoded by the *ycdHIyceA* operon (13). Zur also represses three genes (*ytiA*, *rpmGC*, and *yhzA)* encoding paralogs of ribosomal proteins (26). The *ytiA* gene encodes an alternative form of L31 lacking zinc. L31 (encoded by *rpmE*) is a

small, zinc-containing protein that associates with the large ribosomal subunit (23). When zinc is limiting in the cell, YtiA is expressed and displaces L31(RpmE) from the ribosome which is then postulated to liberate zinc for essential cellular functions (1). The RpmGC protein, encoding a zinc-free L33 paralog, is postulated to play a similar role by displacement of one or more of the other two, zinc-containing L33 proteins (encoded by *rpmGA* and *rpmGB*). Finally, the Zur regulated YhzA protein can functionally replace S14, a zinc metalloprotein required for ribosomal assembly. Expression of YhzA provides a "failsafe" mechanism to allow continued ribosome assembly even under severe zinc limitation (24).

Recently, insights have begun to emerge into the functions of the remaining Zur-regulated genes. ZinT is postulated to function in zinc trafficking, but few details are understood. YciA represents a novel class of GTP-cyclohydrolase, an enzyme typically requiring a zinc metal co-factor (11). When there is insufficient zinc to support the catalytic activity of MtrA, a zinc containing GTP-cyclohydrolase essential for folate biosynthesis, derepression of YciA allows continued growth (Sankaran *et al.* submitted for publication). YciC was originally identified as an abundant, membrane-associated protein identified in extracts of *zur* mutant cells. Early studies suggested that a *yciC* mutation further exacerbated the growth defect of a transporter deficient strain under zinc limitation (13). This observation led to the speculation that this protein might play a secondary role in zinc uptake. However, protein sequence comparisons indicate that YciC has similarities with factors implicated in protein metallation reactions, suggesting that YciC may instead be involved as a metallochaperone (19). It is not yet known whether YciC functions as a zinc metallochaperone or perhaps as a chaperone for one or more other metal ions.

Here, we report a series of studies to define the genetic requirements for the zinc-dependent repression of *yciC* transcription. Although also expressed as part of the

Zur-regulated *yciABC* operon, the bulk of *yciC* transcripts initiate from within the *yciB-yciC* intergenic region. We demonstrate that this region contains a $\sigma^A$-dependent promoter and two Zur boxes separated by nearly 200 bp. While either Zur box can mediate zinc-responsive repression of *yciC*, complete repression requires the promoter-proximal C2 box. DNA-binding studies demonstrate that Zur recognizes a minimal operator site (a 9-1-9 inverted sequence motif) that is somewhat larger than that reported for other characterized Fur family members.

## 3. Materials and Methods

**Bacterial strains and growth conditions.** All strains are derivative of the wild-type CU1065 (*trpC*2 *att*SPβ). Strain HB8010 (CU1065 SPβ 8008 *yciC'-cat-lacZ*) was used for WT *yciC* promoter activity as previously described (13). HB8541 (CU1065 SPβ 8508 $P_{yciC}$ΔC1-*yciC'-cat-lacZ*) and HB 8542 (CU1065 SPβ 8509 $P_{yciC}$ΔC2-*yciC'-cat-lacZ*) were created by cloning (see below) to investigate each Zur box individually. *B. subtilis* was grown in LB or in a defined minimal media as previously described (14). Erythromycin (1 μg/ml) and lincomycin (25 μg/ml), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), neomycin (10 μg/ml), and chloramphenicol (5 μg/ml) were used for the selection of various *B. subtilis* strains.

**Selection of spontaneous mutants derepressed for *yciC'-cat-lacZ*.** HB8010 was grown overnight in LB medium containing 5μM Zn and 2μg/ml chloramphenicol. LB medium containing 5μM Zn(II) and 4μg/ml chloramphenicol was inoculated at a 1:20 dilution of the culture and grown overnight. This process was repeated for LB containing 5μM Zn and 7μg/ml chloramphenicol and for LB containing 5μM Zn and 10μg/ml chloramphenicol. Phage were induced and used to transduce CU1065 followed by selection for the phage and screening for loss of repression. Cells were

also cured of phage by incubation at 50° overnight followed by screening for loss of phage-linked antibiotic resistance.

**Construction of mini-Tn10 libraries.** To determine the possible locus of the trans-acting mutations transposon insertion mutagenesis was used to identify mutants derepressed for *yciC'-cat-lacZ* expression. Libraries of random mini-Tn10 insertions in HB8010 were constructed using plasmid pIC333 (30), which contains a ColE1 origin and a thermosensitive origin of replication for gram-positive bacteria, which is inactive at above 35°. HB8010 were transformed with pIC333 and transformants selected for spectinomycin resistance on LB plates incubated at 30° overnight. Single colonies were inoculated to LB-erythromycin, lincomycin (MLS) and spectinomycin (Spc) plates and grown overnight at 30°. 5ml of LB/MLS/Spc were inoculated at a 1:100 dilution of the overnight culture and grown 3 hours at 30° followed by 4 hours growth at 37°. 1:500 dilutions of this culture were plated on LB and LB/Spc. Frozen permanents were made from approximately 20% of the remaining culture, while 10% was plated on medium containing chloramphenicol to select for the derepression of *yciC'-cat-lacZ*. The transposition frequency was estimated from the ratio of colonies on selective versus nonselective media and was consistent with the frequency (0.01 to 1%) reported for this system.

**Isolation of mini-Tn10 insertions that derepress *yciC'-cat-lacZ*.** Chromosomal DNA was prepared from derepressed mutants and transformed into strain HB8010 with selection for spectinomycin resistance. Transformants were screened for derepression to determine if the phenotype was linked to the transposon insertion. After verification of linkage, DNA from the derepressed strains was recovered by plasmid rescue. Restriction analysis suggested some plasmids were identical, but five unique plasmids were further characterized by sequencing.

**Construction of a strain merodiploid for *zur*.** HB8519 was constructed using the pXT system, which allows integration by double crossover at the *thrC* locus of the *B. subtilis* chromosome. The plasmid is a derivative of pDG1731 (10), which fuses a xylose-inducible promoter to the gene of interest. Strains were selected for $spc^R$ and screened for $MLS^S$ and threonine auxotrophy. A primer located upstream of the putative ribosome-binding site (shown in italics) and containing an engineered BamHI site shown in bold (5'GTTC**GGATCC**AAAGCGAA*AAGGGG*G 3') was used in conjunction with a primer located downstream of a putative hairpin terminator (5'CGCGT**GAATTC**CTGAAAAAGGAGCCC 3') with an engineered EcoRI site shown in bold to amplify *zur* with Pfu polymerase. The resulting PCR product was digested with BamHI and EcoRI and cloned into pXT (10) digested with the same enzymes. The resulting plasmid was linearized with ScaI and used to transform competent HB1000 cells. The resulting strain (HB8520) was transduced with SPβ-8008 (*yciC'-cat-lacZ*).

**Selection for derepressed mutants in a *zur* merodiploid strain.** Selection and screening for derepressed mutants in the merodiploid *zur* strain was carried out on LB plates containing 10μM Zn, 40μg/ml Xgal, 20mM xylose, and increasing concentrations of chloramphenicol (2, 4, 6, 8, and 10μg/ml). Four phage-linked mutants were isolated that were derepressed for *yciC'-cat-lacZ* expression. Chromosomal DNA was isolated from these strains and used as a template for PCR with primers #533 and #366. The resulting PCR products were characterized by sequencing using #533(GTACATATTGTCGTTAGAAC) forward primer located upstream of MCS in pJPM122 (29) and #366 (ACTCTCCGTCGCTATTGTAACCAG) reverse primer located in *cat* gene of pJPM122

**Construction of C1- and C2-*cat-lacZ* fusions.** Primer 240 (5'ctgaagcttccagatgcgaaatgggtata 3') and primer C2rev

(5’ggcttatcattgtctgtggatccgtg3’) were used to clone a fragment containing only the C2 box and associated promoter into pJPM122 to make promoter-*cat-lacZ* fusions in the SPβ phage. Primer C1for (5’tttaaagctttagaaatcgggcgg 3’) and primer 241 (5’aaaacaacattgctgaagacgattggatccg 3’) were used to clone a fragment containing the previously proposed promoter elements and C1 box into pJPM122. These constructions were used to test β-galactosidase activity.

**Construction of deletions of C1 or C2 in the full length *yciC* promoter**. Primer delC1rev (5’AAA**CTGCAG**ACTTCGCCGTATGTACAATGG 3’) containing a PstI (bolded) site to replace part of C1 and primer 240 were used to amplify chromosomal DNA. Primer delC1for (5’AAA**CTGCAG**GCACTATTATGAAAAAAATTC 3’) and primer 241 also containing a PstI site were used to amplify chromosomal DNA. The two resulting products were digested with PstI and ligated together. The 450bp fragment was gel purified (Qiagen) and digested with HindIII and BamHI. The product was purified (Qiagen) and ligated to pJPM122 cut with the same enzymes to construct promoter-cat-lacZ fusions. The same process was followed to create the C2 deletion using primer 240 with primer delC2rev (5’AAACTGCAGTACGACTTAAATTGTCTTTTTTCC 3’) and primer delC2for (5’AAACTGCAGTGGCTTATCATTGTCTGTGCA 3’) with primer 241.

**β-galactosidase assays.** Zinc deficiency was achieved as described previously (13). The cultures were grown overnight and cells were harvested to assay β-galactosidase activity as described elsewhere (5, 22).

**Protein purification.** *B. subtilis* Zur protein was expressed in *E. coli* BL21(DE3)/pLysS (13). For purification of Zur, a single colony was grown overnight in LB containing ampicillin (200 μg/ml), and 0.4% (wt/vol) glucose. The overnight culture was used to inoculate a 500-ml LB containing ampicillin (200 μg/ml), and 0.4% (wt/vol) glucose, and the flask was incubated at 37°C with vigorous shaking to

an optical density at 600 nm of 0.8. Isopropyl-β-D-thiogalactopyranoside (1mM final concentration) and Zinc (50μM final concentration) was added and cells continued growth at 30°C. The addition of zinc and the temperature shift significantly increased the soluble portion of Zur. The cells were harvested after further incubation for 4 h. Cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C, and the pellet was stored at ˉ80°C till used. The cell pellet was thawed on ice for 30 min and suspended in 10 ml resuspension solution (50 mM Tris·Cl, pH 8.0/2 mM EDTA, pH 8.0/0.1 mM DTT/1 mM β-mercaptoethanol/100 mM NaCl/1 mM PMSF/5% glycerol), and the cells were broken by sonication. The lysate was clarified by centrifugation at 10,000 rpm for 5 min at 4°C and applied to a heparin column. Bound proteins were eluted with gradient of NaCl (0.05-1 M) in elution buffer (50 mM Tris·Cl pH 8.0, 2 mM EDTA, 0.1 mM DTT, 1 mM PMSF, 5% glycerol). Samples were loaded on 12% SDS-PAGE to identify the fractions that contained Zur. Proteins were then loaded on a Bio-Rad Q2 ion-exchange column via FPLC (Pharmacia). A linear gradient of 0.05-1 M NaCl was used to elute the protein, and then the peak fractions were injected onto a Superdex 200 column (Pharmacia). Purified Zur was stored at -20 °C in TEDG buffer containing 50% glycerol.

**Electrophoretic mobility shift assay (EMSA) of Zur binding**. PCR fragments containing the full *yciC* promoter region, the C1 Zur box, the C2 Zur box, D1.5 deletion or D2.1 deletion were purified from agarose gel and used in EMSA experiments as previously described (13). Synthetic oligonucleotides were synthesized (Integrated DNA Technologies Incorporated) and 30 fmol were end-labeled with [γ-$^{32}$P]ATP. After labeling and removal of the unincorporated label, 30 fmol of the complimentary oligo was added and annealed by incubation at 95°C for 10 mins. followed by transfer to room temperature. Duplex oligonucleotide probes were incubated with Zur and run on a 12% polyacrylamide gel for 45 mins at 100V. $K_d$

values for the full length probes were determined by quantifying the disappearance of the free probe through the program ImageQuant. Percent shift was plotted versus protein concentration and $K_d$ was defined as the concentration of protein required to shift 50% of the DNA probe. For the oligonucleotide studies, relative $K_d$'s were approximated by the appearance of the shifted complex due to the amount of background caused by unshifted single stranded probe.

**Primer extension analysis.** Total RNA was isolated from wild type or *zur* mutant cells using the RNeasy RNA isolation kit (Qiagen). For primer extension analysis, 100μg of total RNA was precipitated with 4 pmol of end-labeled reverse primer and the reverse transcripts generated as described (15, 16). Reverse transcripts were analyzed using 8 M urea 6% PAGE. The PCR product was sequenced using the same primer to index the reverse transcripts.

**5' RACE.** Total RNA was isolated from mid-exponential growing *zur* mutant cells using RNeasy RNA isolation kit (Qiagen). 2μg of isolated RNA was used in the 5' RACE kit (Invitrogen) according to the manufacturer's protocol. Products were sequenced at the Cornell Biotechnology Resource Center.

**Sequence logo creation.** The Zur box sequence logo was created through the alignment of the eight known Zur regulon members of *B. subtilis*, three orthologous genes from *B. amyloliquefaciens* and from *Oceanobacillus iheyensis*. The ClustalW alignment was entered into: http://weblogo.berkeley.edu/ to create the logo (28).

**Nucleotide sequence corrections.** We have identified a single amino acid difference in *B. subtilis* 168 strains relative to the previously reported *zur* sequence (17).The change is in codon 12 from GGA (Gly) to GAA (Glu). This difference is consistent with the conservation of a negatively charged amino acid at this position in other Zur homologs.

Table 3.1 Site directed mutants of the *Bacillus subtilis* Zur box

| Sequence Name | Sequence[1] | Kd | Reference |
|---|---|---|---|
| Consensus | AAATCGTAATnATTACGATTT | 10nM | |
| C1 | cgcagtcAAATCGTAATcATT**CTAT**TTTctagata | 100nM | This work |
| C2 | cgcagtcAA**G**TCGTAA**C**aATTACG**T**TTTctagata | 100nM | This work |
| 9 | cgcagtcA**G**ATCGTAATcATTACGAT**C**Tctagata | 320nM | This work |
| 8 | cgcagtcAA**G**TCGTAATcATTACGA**C**TTctagata | ≥320nM | This work |
| 7 | cgcagtcAAA**G**CGTAATcATTACG**C**TTTctagata | ≥320nM | This work |
| 6 | cgcagtcAAAT**T**GTAATcATTAC**A**ATTTctagata | NB | This work |
| 5 | cgcagtcAAATC**T**TAATcATTA**A**GATTTctagata | NB | This work |
| 4 | cgcagtcAAATCG**G**AATcATT**C**CGATTTctagata | 100nM | This work |
| 3 | cgcagtcAAATCGT**G**ATcAT**C**ACGATTTctagata | NB | This work |
| 2 | cgcagtcAAATCGTA**C**TcA**G**TACGATTTctagata | ≥320nM | This work |
| 1 | cgcagtcAAATCGTAA**G**c**C**TTACGATTTctagata | 320nM | This work |
| 9-1-9 | cgcagtc**G**AATCGTAATcATTACGATT**C**ctagata | 10nM | This work |
| 8-1-8 | cgcagtc**GC**ATCGTAATcATTACGAT**GC**ctagata | ≥320nM | This work |
| 7-1-7 | cgcagtc**GCG**TCGTAATcATTACGA**CGC**ctagata | NB | This work |
| 6-1-6 | cgcagtc**GCGCC**GTAATcATTACG**GCGC**ctagata | NB | This work |
| mrgA(1) | AAATC**A**TAATTATTA**T**GATTT | 150nM | (12) |
| mrgA(2) | AAATCGTAATTATTACGATTT | 20nM | (12) |
| feuA(1) | AA**T**TC**A**TAATA**G**TTA**T**GA**A**TT | 1 μM | (12) |
| feuA(2) | AA**T**TCGTAATA**G**TTACGA**A**TT | 150 nM | (12) |

[1] The Zur box sequence is capitalized and bases which differ from consensus are in bold.

## 4. Results and Discussions

**Genetic identification of factors mediating repression of *yciC*.** We previously demonstrated that a *yciC'-lacZ* reporter fusion is tightly repressed during growth in medium containing zinc (13). In addition, we noted the presence of a candidate Zur box element preceding *yciC* with an overlapping sequence resembling a $\sigma^A$-type promoter (13). Since the sequence requirements for DNA-binding by Zur are not well characterized, we initially considered a genetic approach to define critical components of the Zur box. In analogous studies, we selected for chloramphenicol resistance using an *mrgA'-cat-lacZ* reporter fusion and identified point mutations and small deletions that defined important bases within the Per box element (6). We reasoned that a similar genetic approach, using a *yciC'-cat-lacZ* reporter fusion (see Materials and Methods), might identify bases within the Zur box critical for recognition by the Zur protein.

In an initial study, we selected spontaneous mutants that were derepressed for *yciC'-cat-lacZ* expression. Since the *yciC'-cat-lacZ* reporter is carried on the SPβ prophage, cis-acting mutations are easily identified by virtue of being linked to the phage DNA in transduction experiments. Our initial studies failed to identify any cis-acting mutations. In contrast, we found that 8 strains cured of phage were still derepressed when a *yciC'-cat-lacZ* fusion was reintroduced, consistent with the presence of a trans-acting mutation. When the selection was repeated with mini-*Tn*10 mutagenesis using plasmid pIC333 (30) all sequenced transposants had insertions within the *zur* gene.

To significantly reduce the frequency with which *zur* mutants were recovered, a merodiploid *zur* strain was created. Using this strain, two unique *cis*-acting mutations were obtained and both contained large deletions (deletion mutants 1 and 2; see Figure 3.1C). Surprisingly, these deletions included both the Zur box and the

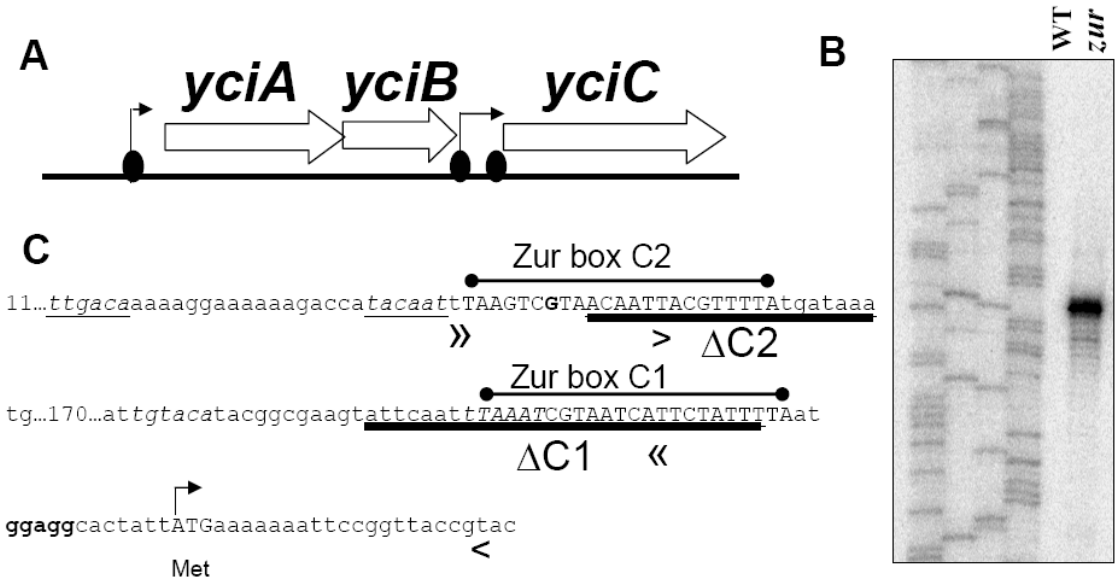


Figure 3.1 - **A.** The *yciABC* complex operon. Open reading frames are indicated by open arrows, promoter sites by bent arrows, and Zur boxes by filled circles. **B.** Primer extension mapping of the transcription start site of *yciC*. The primer extension product was generated using RNA isolated from either wild type or *zur* mutant cells grown in LB medium. The sequence ladder was generated by PCR cycle sequencing with the same primer. **C.** Partial sequence of the intergenic region between *yciB* and *yciC*. On the left there are 11 bases not shown to the stop codon of *yciB* and 170 bases between the box C2 and C1 regions. Single arrowheads frame the bases missing in deletion mutant 1 (276 bases) and double arrowheads frame the missing bases of deletion mutant 2 (246 bases). Black bars represent the area which has been deleted to create strains possessing only one functional Zur box: ΔC1 (deletion of 29 bases) and ΔC2 (deletion of 21 bases). The ribosome binding site and transcription start site are in bold while the -10 and –35 promoter elements are underlined and in italics. The originally proposed promoter for *yciC* in the downstream region is italicized. The start codon (ATG) for *yciC* is indicated.

previously proposed promoter (13). These findings led to the discovery of a second candidate $\sigma^A$ promoter and a second potential Zur box element in the *yciB-yciC* intergenic region. In retrospect, the deletion mutations removed both of the Zur box elements thereby placing the upstream *yciC* promoter region adjacent to the *cat-lacZ* reporter fusion.

**Transcriptional arrangement of the *yciABC* complex operon.** The Zur-regulated *yciA*, *yciB*, and *yciC* genes are clustered on the chromosome (Fig. 3.1A). Previously, we mapped a transcriptional start site preceding *yciA* (14). Northern blot experiments demonstrated that ~ 85% of the *yciC*-hybidizing transcripts initiated from within the *yciB-yciC* intergenic region, while a small fraction corresponded to read-through transcription from the upstream *yciA* promoter (and hybridized with a *yciA* probe) (data not shown).

In light of our genetic results, we hypothesized that the abundant monocistronic *yciC* transcript initiated from the candidate promoter 260 bp upstream of the *yciC* start codon. Indeed, a strong transcript from this promoter was detected by primer extension analysis when using RNA extracted from the *zur* mutant, but not from wild-type cells (Fig. 3.1B). To determine if there was any transcription from the downstream promoter-like sequence, nested primers were used to probe for a transcription start site using 5'RACE experiments. The only strong and reproducible start site observed in numerous 5' RACE experiments corresponded to initiation from the G residue at the upstream promoter consistent with the primer extension results. There are no conserved open reading frames encoded within this leader region, and we therefore suggest that the *yciC* gene is expressed with an unusually long 5'-untranslated region. This leader region contains both the originally noted Zur box (designated C1) and the upstream promoter proximal Zur box (C2). Note that in our

previous description of the Zur regulon we included the correct *yciC* promoter based on the data now presented (14).

**Both the C1 and C2 Zur boxes mediate zinc responsiveness.** The selection of deletion mutations removing most or all of the C1 and C2 Zur boxes suggested that removal of both sites was necessary to bypass Zur-mediated repression. As predicted, these deletion mutants displayed full promoter activity (as judged by *lacZ* fusion assays), and this activity was no longer repressible by added zinc (data not shown). Moreover, purified Zur was unable to bind to DNA fragments derived from the *yciB-yciC* intergenic regions carrying these large deletions as judged by EMSA experiments using up to 400 nM added Zur (data not shown). Together these results suggest that inactivation or deletion of either box alone was not sufficient for derepression of *yciC* and therefore for the generation of chloramphenicol resistance under our selection conditions.

To assess the relative contributions of the C1 and C2 Zur boxes to Zur-mediated repression of *yciC*, we generated strains in which either box was individually inactivated. The first construct (denoted as ΔC2) contained the downstream box (C1 box) with a deletion spanning over half of the upstream box (C2 box), while leaving intact the –10 region and sequences spanning the transcription start site of the upstream promoter. The second construct (ΔC1) removed the C1 box (Figure 3.1C). When analyzed in the context of *lacZ* reporter fusions, these three constructs all responded to added zinc, but with varying efficiencies (Fig. 3.2). Both the WT and ΔC1 constructs show full repression at ≥1μM added zinc. However, overall promoter activity (as judged by Miller Units) was decreased by 75% in the ΔC1 construct for reasons not yet clear. The ΔC2 construct was impaired in zinc responsive repression with only a 90% reduction in β-galactosidase activity in the presence of 1 μM zinc. Full repression of the *yciC* promoter was not obtained even with 10 μM added zinc.

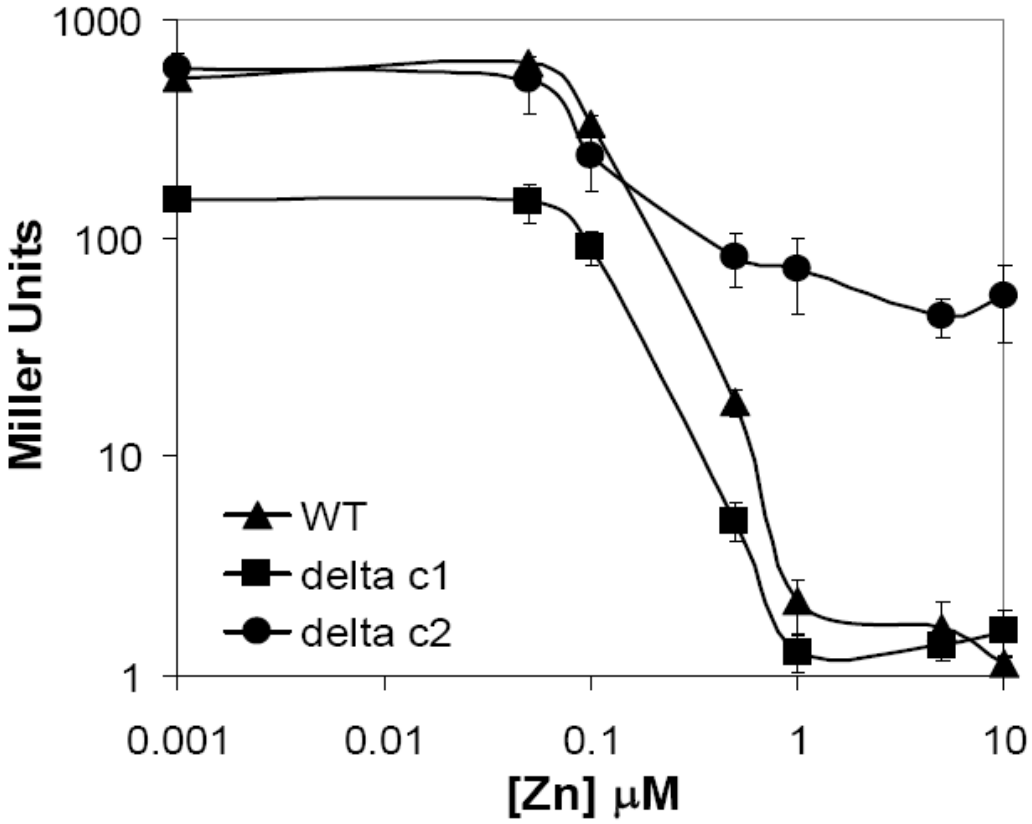


Figure 3.2 Zinc-dependent repression mediated by the C1 and C2 Zur boxes of *yciC*. Promoter activity of the WT *yciC* promoter (filled triangles) is compared with fusion constructs containing either ΔC1 (filled squares) or ΔC2 (filled circles) as a function of added zinc. Data shown are representative of experiments performed in at least five independent trials each time using biological triplicates for each point.

Interestingly, promoter activity of the ΔC2 construct was at wild type levels (as judged by Miller Units) and the level of added zinc where repression is first observed matched that of the WT and ΔC1 constructs. It should be noted that the difference in promoter activity of the constructs should not be due to effects on RNA stability since both constructs contain the WT ribosome binding site and $\sigma^A$ promoter elements.

To further elucidate the relative roles of the C1 and C2 Zur boxes in zinc-mediated repression, we used EMSA experiments to test the affinity of Zur for each Zur box. Zur bound to a fragment containing both Zur boxes with an estimated $K_d$ of 6 nM (Fig 3.3). The fragment containing only the C2 box bound Zur with slightly lower affinity (estimated $K_d$ 9 nM), consistent with the ability of this site to mediate a near wild-type response to added zinc. In contrast, the downstream C1 Zur box fragment bound Zur with slightly lower affinity (estimated $K_d$ 13 nM).

Taken together, these results indicate that the C2 box is sufficient for complete repression by Zur. However, either Zur box can mediate repression with comparable sensitivity to added zinc. The C1 Zur box alone allows significant, albeit not complete, repression of the *yciC* promoter (90% repression). Additionally, our genetic experiments confirm that both boxes need to be deleted to achieve full derepression of a *yciC-cat-lacZ* reporter strain.

Several possible models could account for the role of these two Zur boxes in repression. We currently favor the hypothesis that Zur binds at both sites and each site can function independently. In this model we envision the upstream site accounts for the bulk of the repression, but any RNA polymerase that initiates transcription will ultimately be impeded in elongation by Zur bound at the downstream C1 site. The ability of the C1 site to mediate partial repression is apparent from analysis of the ΔC2 construct. In this construct, elongating RNA polymerases will presumably stall upon

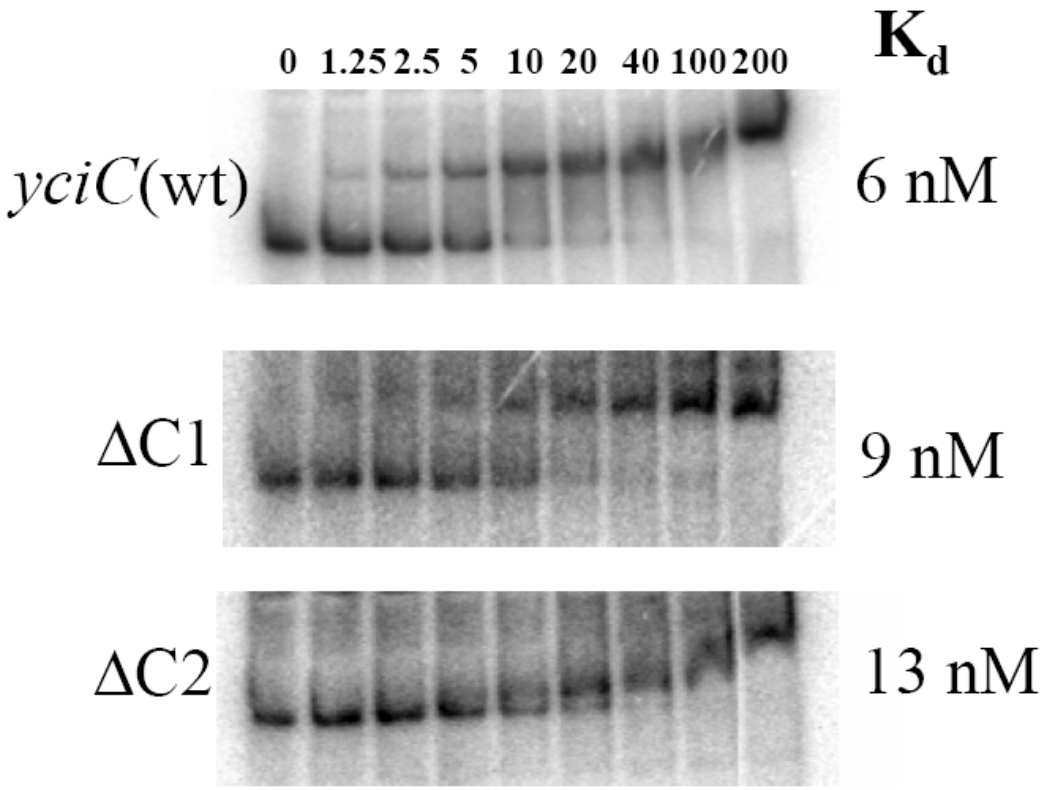


Figure 3.3 Binding of Zur to the *yciC* regulatory region. EMSA experiments with labeled PCR probes containing each of the *yciC* promoter constructs were used to measure Zur binding affinity. The concentration of Zur in each reaction is shown in nM. The WT promoter fragment has a binding affinity in the range previously reported ($K_d$ ~5 nM) (14). Deletion of the upstream C2 box decreases Zur affinity only slightly, and deletion of the downstream C1 box decreases affinity ~2 fold when compared to WT.

encountering Zur bound at C1 and some or all may be dissociated by the action of Mfd (28). However, transient dissociation of Zur, or displacement by elongating RNA polymerase, may still allow significant expression of *yciC*. Alternatively, some Fur proteins are known to polymerize on DNA to create extended arrays of bound repressor (18), and it is possible that this also occurs between the C1 and C2 sites. We disfavor this idea, however, since Zur has not been observed to polymerize on DNA and our EMSA results do not show evidence for high molecular weight complexes. Finally, it is possible that sites C1 and C2 function cooperatively. While the EMSA results do suggest that the WT construct has a slightly higher affinity for Zur than either box alone, the *in vivo* data show that the C2 box is sufficient for full repression. Thus, if there is cooperativity in this system, it is modest and not critical for mediating repression.

**Zur binds to a conserved 9-1-9 inverted repeat**. We previously reported that Zur binds to DNA sites with similarity to those recognized by two other Fur paralogs, Fur and PerR (14). The Zur box differs from the Fur and Per boxes at positions 5 and 6 within each half site and also displays conservation of bases at flanking positions not strongly conserved in Fur and Per boxes (12) (Fig. 3.4A; positions 8,9, and 10). Indeed, sequence searches using this conserved motif have been useful in providing insights into the Zur regulon in many different bacterial systems (26).

To identify bases critical for Zur binding we generated a set of duplex oligonucleotides systematically altered at each position of the inverted repeat. The consensus WT sequence (as determined from the Sequence Logo; Figure 3.4a) is a perfect 10-1-10 inverted repeat while the C1 and C2 Zur boxes each contain several bases that differ from consensus (Table 3.1). The perfect consensus sequence shows high affinity binding by Zur with an estimated $K_d$ of 10 nM. In contrast, the oligonucleotides containing the C1 and C2 Zur boxes have similar, but reduced

affinity ($K_d$ ~100 nM). We note that the affinity measured for the C1 and C2 Zur boxes within these duplex oligonucleotides is less than that measured with larger DNA fragments, suggesting that flanking regions also contribute to the observed binding affinity. Nevertheless, in both experiments, the C1 and C2 boxes are found to have similar affinities for Zur.

Our mutagenesis studies indicate that symmetric mutations in each half site at positions 5 or 6 completely abrogate Zur binding, consistent with previous studies(12). In addition, symmetric mutations at all positions between 2 and 9 significantly reduce Zur binding (Table 3.1). Together, these results support the important role in DNA-binding affinity inferred from sequence conservation. In addition, these results demonstrate that Zur requires a 9-1-9 inverted repeat element: decreasing the extent of the inverted repeat in the 8-1-8 and 7-1-7 constructs drastically reduces binding affinity. In contrast, alteration at position 10 (in the 9-1-9 construct; Table 3.1) did not reduce Zur binding. The importance of positions 8 and 9 contrasts with published results for PerR and Fur which demonstrate that the 7-1-7 core motif is sufficient for high affinity binding by these proteins (2, 12). It is interesting to note, however, that recent analysis of DNA sequence conservation patterns in several genomes suggests that the optimal Fur-binding sequence in Gram negative bacteria is best modeled as a 9-1-9 motif (7), rather than the 7-1-7 motif noted for *B. subtilis* (2).

When the sequences of the C1 and C2 boxes are considered in light of these binding studies it is apparent that neither site is optimal. Both sites contain multiple mismatches that likely account for their reduced affinity relative to the consensus sequence. Since we do not have a complete set of all possible single mutations, it is not possible to infer if some substitutions are more favorable than others. It is interesting to note, however, that the C1 box still contains one perfect half-site and the C2 box contains one half-site with only one change from consensus.

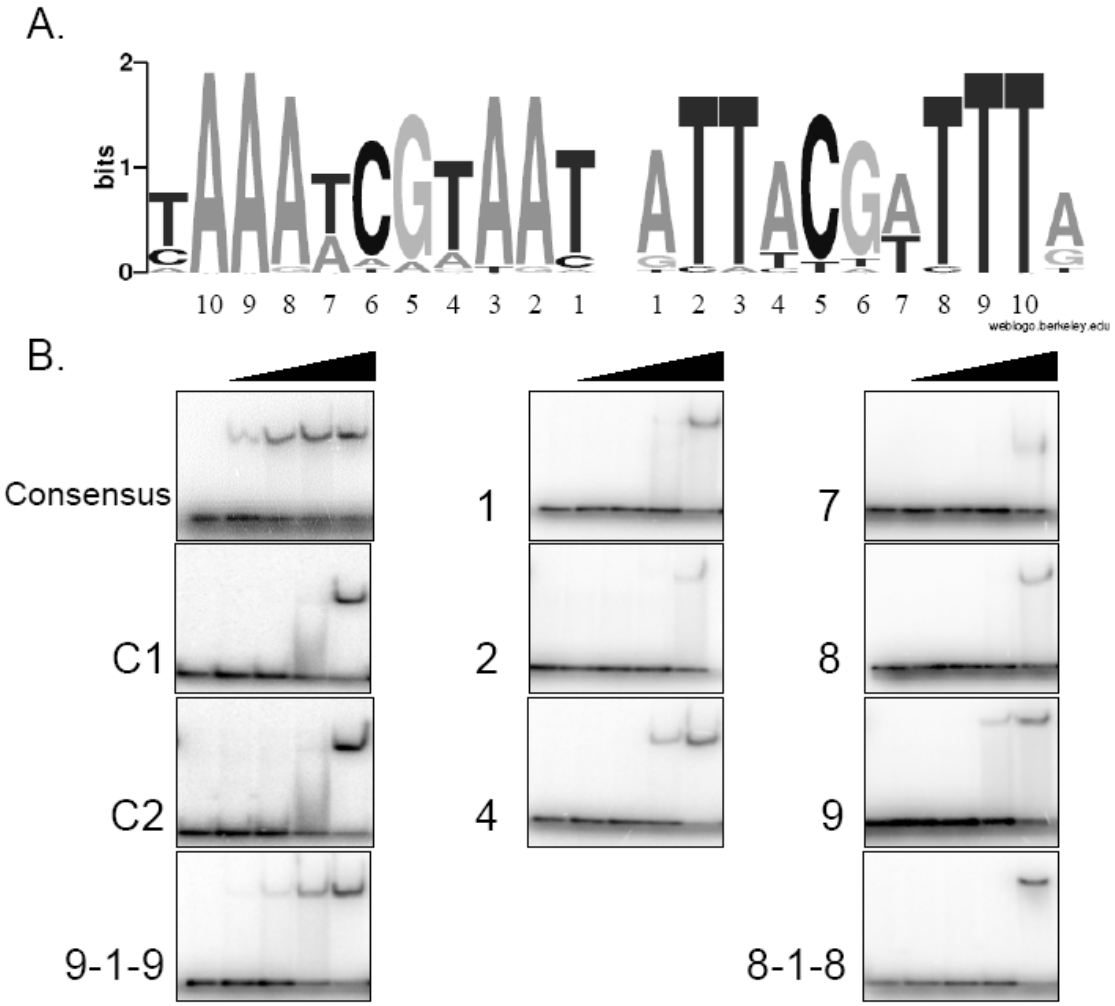


Figure 3.4 DNA sequence requirements for Zur-DNA binding. **A.** Sequence Logo illustrating conservation of bases within aligned Zur box sequences. **B**. Representative EMSA experiments with duplex oligonucleotides and added Zur protein (0, 5, 20, 80, and 320 nM). Only those oligonucleotides where shifts were observed are shown. All shifts were done in the presence of 50 μM added zinc. $K_d$ values (see Table 1) were estimated as the concentration of Zur at which half maximal binding was observed.

## 5. Concluding Remarks

We began this work with the intent of using a genetic selection to identify bases critical for the Zur-mediated repression of *yciC* with the expectation that this might provide insights into Zur-operator DNA interaction. Unexpectedly, the *yciC* gene was found to be regulated by two Zur boxes (C1 and C2). As a result, derepression of this locus requires large deletions spanning both Zur boxes. Together, these two sites allow for a very tight repression of *yciC* which encodes a highly abundant protein postulated to function as a metallochaperone. The significance of the long, untranslated leader region preceding *yciC* is not clear. Metal-responsive riboswitches have recently been described (8), so it is formally possible that this region could function in sensing zinc or a zinc complex within the cell. However, this seems unlikely since we do not detect any zinc responsiveness for a *yciC-lacZ* fusion in a *zur* mutant strain and neither the sequence nor structure of this intergenic region in conserved in other Bacilli. In many related Gram positive bacteria, the *yciA* and *yciC* genes are clustered, but there is no obvious *yciB* homolog. The functions of the *yciC* and *yciB* genes clearly require additional study.

Next, we turned to DNA-binding (EMSA) studies using synthetic duplex oligonucleotides to define the critical determinants of the Zur-DNA interaction. Our results confirm the roles of the conserved bases within the Zur box and demonstrate that a 9-1-9 inverted repeat provides the minimum site needed for high affinity binding. This contrasts with the related results for *B. subtilis* Fur and PerR (2, 12). To date, there are no structures available for protein-DNA complexes for proteins of the Fur family, so the structural basis for the fine-tuning of protein-DNA specificity is not yet clear.

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# CHAPTER 4

# CONTRIBUTIONS OF ZUR-CONTROLLED RIBOSOMAL PROTEINS TO GROWTH UNDER ZINC STARVATION CONDITIONS

## 1. Summary

Maintaining intracellular zinc levels is critical, because zinc serves as a cofactor for many required enzymes and is toxic in excess. *Bacillus subtilis* Zur, a Fur family repressor, controls the zinc starvation response including two ribosomal proteins (r-proteins) paralogous to L31 and S14. Biochemical analyses suggest that Zur-controlled r-proteins (which lack CxxC metal-binding motifs) may functionally replace their cognate zinc-requiring proteins during zinc limitation. We here demonstrate that Zur-regulates expression of an additional r-protein paralog, RpmGC (L33c), and, using strains defective in zinc uptake, we investigate the physiological contributions of all three Zur-regulated r-proteins. In the 168 lineage, *rpmGC* is a pseudogene containing a frameshift mutation. Correction of this mutation allows expression of a functional L33c that can suppress the poor growth phenotype of an *rpmGA rpmGB* (encoding L33a, L33b) double mutant. Similarly, we provide physiological evidence in support of the "failsafe" model (Natori *et al.* Mol. Microbiol. 63:294-307) in which the Zur-regulated S14 paralog YhzA allows continued ribosome synthesis when there is insufficient zinc to support S14 function. The L31 paralog YtiA can replace L31 and complement the growth defect of an *rpmE* mutant (Nanamiya *et al.* Mol. Microbiol. 52:273-83). We show that, under zinc starvation conditions, derepression of YtiA significantly increases the growth of cells in which pre-existing ribosomes carry, as the sole L31 protein, RpmE (containing zinc) but not if they carry YtiA (which lacks zinc). These results support a direct and physiologically relevant role for YtiA in mobilizing zinc from ribosomes.

## 2. Introduction

Zinc is a required metal cofactor for many proteins and thus controlling zinc levels within the cell becomes one of utmost importance. *Bacillus subtilis* Zur, a Fur family member, is responsible for controlling the response to zinc starvation. Its regulon includes a high affinity zinc uptake system (an ABC transporter encoded by the *ycdHI-yceA* operon), the complex *yciAB-yciC* operon encoding a putative metallochaperone (YciC), *zinT*, and several ribosomal protein (r-protein) paralogs (7, 17).

As anticipated from their roles in ribosome assembly and function, ribosomal-proteins (r-proteins) are highly conserved and usually encoded by essential genes (9). However, bacterial genomes often contain duplicate copies of the genes encoding some or all of the r-proteins L36, L33, L31 and S14. Interestingly, the duplicated proteins fall into two groups: those which contain a zinc binding motif (two CxxC motifs; designated at C+) and those which do not (designated C-) (11). Bioinformatic analyses suggest that the non zinc-containing proteins are likely to be preferentially expressed under zinc-limiting conditions: they are associated with predicted binding sites for Zur or functionally analogous zinc-sensing transcription factors (17). Thus, it was hypothesized that the constitutively expressed C+ r-protein paralogs use zinc as a co-factor and, in times of zinc depletion, they are replaced by their Zur-controlled C-counterparts (11, 17).

With the re-sequencing of the *B. subtilis* strain 168 genome, it appears there are five duplicated pairs of r-proteins (2). Three of the duplicated genes are under the control of Zur and are presumed to facilitate adaptation to zinc limiting conditions (17). The functions of the others are unknown. The Zur-regulated L31 paralog, YtiA, replaces the C+ protein, RpmE, in ribosomes isolated from cells grown under zinc-limiting conditions (1). YhzA, a Zur-regulated S14 r-protein paralog, was postulated

to allowed continued ribosome synthesis in the absence of available zinc (15). Unlike L31, which is surface-exposed and loosely associated with the ribosome, S14 is buried deep within the ribosome and is required for *de novo* assembly (15). A gene for a third r-protein paralog, *rpmGC*, has been postulated based on its association with a Zur box-like regulatory sequence (17), but the function of this gene is unknown and, in *B. subtilis* 168 strains, *rpmGC* is a pseudogene.

The Zur-regulated C- proteins may function solely to maintain the proper assembly or functioning of the ribosome during zinc limitation. In addition, or alternatively, these proteins may have extra-ribosomal functions. Specifically, it has been speculated that displacement of L31 (and possibly L33) from the ribosome by the C- paralogs might function to 'mobilize' stored zinc (1, 12). According to this latter model, the major function of L31 in the cell might be, in fact, to provide a mobile store of zinc ions rather than as a functional component of the ribosome (1, 13). It has been noted, for example, that L31 (unlike most r-proteins) is not essential and is only loosely associated with the ribosome. In contrast, S14 is essential for the assembly of the ribosome: replacement of this protein by the Zur-regulated YhzA protein was postulated to provide a "fail-safe" mechanism to maintain de novo ribosome synthesis under zinc limiting conditions (15).

Here, we have used strains deficient in high affinity zinc uptake to test the contribution of the Zur-controlled r-proteins to zinc nutrition and/or continued ribosome function. We demonstrate that *rpmGC* (encoding the L33 paralog designated L33c) is Zur-regulated and derepression of L33c suppresses the growth defect of an *rpmGA rpmGB* double mutant, but only if the frameshift mutation in *rpmGC* is corrected. Expression of L33c does not confer a significant growth advantage in zinc-limiting conditions, which argues against a key role in zinc mobilization. In contrast, derepression of the L31 paralog, YtiA, does confer a significant growth advantage

under zinc limitation, but only if the pre-existing ribosomes contain RpmE (containing zinc) and not if they contain YtiA (lacking zinc). These results provide physiological evidence in support of the hypothesis that YtiA mobilizes a physiologically relevant pool of zinc from the ribosome to facilitate growth under conditions of severe zinc deprivation.

### 3. Materials and Methods

**Bacterial strains and growth conditions.** All strains (Table 4.1) are derivatives of the wild-type CU1065 (*trpC2 att*SPβ). *B. subtilis* was grown in LB or in a defined minimal medium as previously described (7). Erythromycin (1 μg/ml) and lincomycin (25 μg/ml), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), neomycin (10 μg/ml), and chloramphenicol (5 μg/ml) were used for the selection of various *B. subtilis* strains. Zinc starvation minimal media (ZSMM) was prepared with chelexed and filter-sterilized stocks of all non-metal containing components and ultrapure filter sterilized metal stocks containing 40 mM potassium morpholinepropanesulfonate (MOPS) (adjusted to pH 7.4 with KOH), 2 mM potassium phosphate buffer (pH 7.0), glucose (2%, wt/vol), $(NH_4)_2SO_4$ (2g/liter), $MgSO_4$ -$7H_2O$ (0.2 g/liter), trisodium citrate $2H_2O$ (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), and 80 nM $MnCl_2$ (5). To more readily generate zinc starvation conditions in this medium we also used strains deleted for the high affinity zinc uptake ABC transporter (encoded by the *ycdHIyceA* operon) and the proposed metallochaperone encoded by *yciC*. Strains were pre-cultured overnight in LB followed by a 1/100 dilution into ZSMM. Once cultures reached mid-log they were spun down, resuspended in 10 mM EDTA to remove any loosely associated metals from the cell wall and then rinsed twice with fresh ZSMM to remove the EDTA. All growth curves were performed using a Bioscreen C Machine (Growth Curves USA). Cultures were grown at $37^0$C

Table 4.1 – *Bacillus subtlis* r-protein characterization strain genotypes

| Strain | Genotype |
|---|---|
| HB 6865 | CU1065 *ycdH::cm citM::tet* |
| HB 6866 | CU1065 *ycdH::cm yciC::kan* |
| HB 6867 | CU1065 *ycdH::cm zinT::spc* |
| HB 6868 | CU1065 *ycdH::cm yciC::kan citM::tet* |
| HB 6869 | CU1065 *ycdH::cm zinT::spc citM::tet* |
| HB 6870 | CU1065 *ycdH::cm yciC::kan zinT::spc* |
| HB 6871 | CU1065 *ycdH::cm yciC::kan citM::tet yciC::kan zinT::spc* |
| HB 6880 | CU1065 *ycdH::cm yciC::kan yhzA::spc* |
| HB 6882 | CU1065 *ycdH::cm yciC::kan ytiA::tet* |
| HB 6883 | CU1065 *ycdH::cm yciC::kan ytiA::tet yhzA::spc* |
| HB 6888 | CU1065 *ycdH::cm yciC::kan rpmE::mls* |
| HB 6889 | CU1065 *ycdH::cm yciC::kan rpmE::mls ytiA::tet* |
| HB 6916 | CU1065 *rpmGA::tet rpmGB::cm rpmE::spc* |
| HB 6918 | CU1065 *ycdH::cm yciC::kan thrC*::*rpmGC-mls* |
| HB 6919 | CU1065 *ycdH::cm yciC::kan ytiA::tet thrC*::*rpmGC-mls* |
| HB 6920 | CU1065 *ycdH::cm yciC::kan yhzA::spc thrC*::*rpmGC-mls* |
| HB 6921 | CU1065 *ycdH::cm yciC::kan ytiA::tet yhzA::spc thrC*::*rpmGC-mls* |
| HB 6972 | CU1065 *ycdH::cm yciC::kan rpmE::mls zur::spc* |
| HB 6975 | CU1065 *ycdH::cm yciC::kan rpmE::mls ytiA::tet zur::spc* |
| HB 6976 | CU1065 *rpmGA::tet rpmGB::cm zur::kan* |
| HB 6983 | CU1065 *ycdH::cm yciC::kan rpmE::mls ytiA::tet amyE::rpmE-spc* |
| HB 6984 | CU1065 *ycdH::cm yciC::kan rpmE::mls ytiA::tet amyE::*$P_{rpmE}$ *ytiA-spc* |
| HB 8250 | CU1065 *rpmGA::tet rpmGB::cm thrC*::*rpmGC-mls* |
| HB 8251 | CU1065 *rpmGA::tet rpmGB::cm zur::kan thrC*::*rpmGC-mls* |
| HB 8252 | CU1065 *rpmGA::tet rpmGB::cm rpmE::spc thrC*::*rpmGC-mls* |
| HB 8253 | CU1065 *rpmGA::tet rpmGB::cm rpmE::spc zur::kan thrC*::*rpmGC-mls* |
| HB 8608 | CU1065 *rpmE::mls* |
| HB 8644 | CU1065 *rpmGA::tet rpmGB::cm* |

with shaking and normalized to a given starting OD (experiments presented range from a starting OD of .005 to .03) after treatment with EDTA and ZSMM washings. Zinc limitation during growth is correlated with both an increase in the lag phase and a slower growth rate. Although variability in the duration of the lag phase is often observed between experiments (presumably reflecting the efficiency of removal of zinc and other cations from the cell wall by EDTA), the relative behavior of strains grown in parallel was highly reproducible.

**Northern Blot.** 7 μg of RNA from WT and HB8604 were run on a 1% agarose gel in the presence of formaldehyde and blotted on Zeta-Probe membrane (Bio-Rad). To construct the *rpmGC* probe, primers 1798 (cggcaagctttgcactgaaacgg) and 1799 (tgtttcacggtgaaggg) were used to create a 150 bp PCR fragment which was subsequently end labeled with $\alpha$-$^{32}$P by polynucleotide kinase (Epicenter) and purified using NucAway columns (Ambion) according to the manufacturer's instruction. Membranes were prehybridizied in ULTRAhyp buffer (Ambion) at 42$^{o}$C for 1 h and then hybridized overnight at 42$^{o}$C. Membranes were washed twice with 2X SSPE for 5 minutes at room temperature and then washed twice with 0.1X SSPE at 42$^{o}$C for 15 minutes. Membranes were visualized on a Storm 840 phosphoimager (Molecular Dynamics).

**Electrophoretic mobility shift assay (EMSA) of *rpmGC* promoter fragment.** PCR fragments containing the *rpmGC* promoter region and a promoter region not known to bind Zur were created by PCR, end labeled with $\alpha$-$^{32}$P by polynucleotide kinase (Epicenter), purified using NucAway columns (Ambion) according to the manufacturer's instruction, and used in EMSA experiments as previously described (6).

**Determining zinc content of the ribosomes.** Ribosomes were purified as previously described (14) from 750mls of mid-log LB cultures. Ribosome preparations were

quantified by UV-spec absorbance ($1A_{260}$=26nM of 70S) and 4-(2-pyridylazo) resorcinol (PAR) was used to determine zinc content of the preparations. Under our experimental buffer conditions (PAR Buffer - 40mM Tris pH 8.0, 5% glycerol, .5% SDS) the absorption maximum of the $Zn^{2+}$-PAR complex was observed at 494 nm and had a linear relationship to zinc concentrations from 0-8μM. 3μM of purified ribosomes were placed in PAR buffer with 0.1mM of PAR and boiled for 15 minutes. After boiling, the absorbance at 494nM was read and the total amount of released zinc calculated.

## 4. Results and Discussion

**Zur-regulation of *rpmGC*.** Bioinformatics studies have revealed that a small subset of r-proteins are duplicated in many bacterial species and that one of the pair invariably contain a Zn ribbon motif (11). In a subsequent study, Panina and co-workers (17) extended this initial observation by showing that genes encoding non-zinc containing r-proteins contained binding sites in their promoter regions for Zur or a related zinc-sensing regulator. This led them to hypothesize that Zur-regulated r-proteins allow the cell to respond to zinc limitation by synthesizing alternative r-proteins that lack a zinc metal co-factor requirement (17). In *Bacillus subtilis*, they identified three Zur-controlled r-proteins (see Table 4.2), two of which have been further characterized (1, 14, 15).

We first sought to explore the possible role(s) of the third postulated Zur-controlled r-protein, L33c, encoded by the *rpmGC* gene (Figure 4.1B). However, a frameshift mutation is present in the putative *rpmGC* gene of *B. subtilis* including strains W168, JH642, and the “undomesticated” strain NCIB3610. In contrast, in closely related *Bacillus* strains (e.g. *B. amyloliquefaciens* and *B. licheniformis*) *rpmGC* encodes a full-length L33 protein. This suggests that this mutation occurred early in

Table 4.2 - *Bacillus subtilis* r-protein ribosomal gene duplications

| | **L31** | **L33** | **S14** |
|---|---|---|---|
| **C+ protein** | *rpmE* | *rpmGA,GB* | *rpsN* |
| **C- protein (Zur controlled)** | *ytiA* | *rpmGC* | *yhzA* |

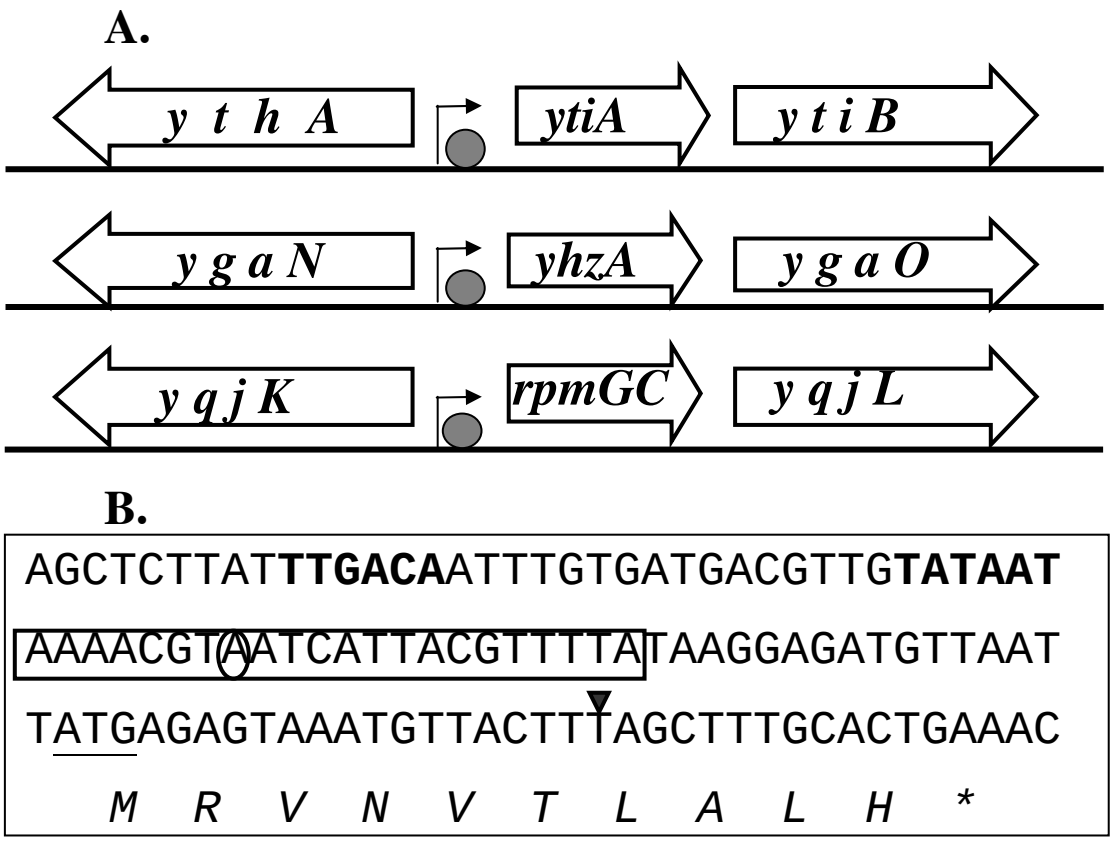



Figure 4.1 A. Genomic context of Zur controlled C- proteins. The bent arrow and filled circle shows the promoter and Zur box respectively. B. Detailed view of *rpmGC* promoter region (for *ytiA* and *yhzA* promoter information see refs 13, 14 respectively). -35 and -10 regions are bolded, Zur operator is depicted by a box and the start site of transcription (as determined by 5' RACE) is circled. Initiating codon is underlined and the site of the frameshift mutation is noted by the filled triangle. Translated message produces a truncated product that terminates four amino acids downstream from the frameshift mutation (in italics).

the lineage leading to current laboratory strains of *B. subtilis* (21). The *B. subtilis rpmGC* pseudogene is located between *yqjK* and *yqjL* (Figure 4.1A). Correction of the frameshift mutation reveals a gene encoding a 49 amino acid protein (L33c), which is over 90% identical to the corresponding *B. licheniformis* L33 C- protein (Figure 4.2A). L33c is also highly similar to the two C+ L33 proteins of *B. subtilis* (RpmGA and RpmGB) (Figure 4.2B) although it lacks CxxC zinc-binding motifs, as noted by Panina *et. al.* (17).

To test whether *Zur regulates rpmGC* we analyzed RNA from WT and *zur* mutant cells in a Northern blot analysis. A transcript corresponding in size to the predicted *rpmGC* transcription unit was only detected in the *zur* mutant strain (Figure 4.2C). Using the same RNA samples, we determined the transcription start site of *rpmGC* by 5'RACE (Figure 4.1B). As expected for a Zur-regulated gene, purified Zur bound with high affinity to the *rpmGC* promoter ($K_d$<5nM) in an EMSA analysis (Figure 4.2D).

**L33c can complement cells deficient for L33a/L33b.** To test whether L33c (encoded by *rpmGC*) is a functional protein, we repaired the frameshift mutation (denoted as *rpmGC*$^{fs}$) and integrated the resulting *rpmGC* (denoted as *rpmGC*$^{+}$) at an ectopic locus. Cells lacking L33 proteins (an *rpmGA rpmGB* double mutant) displayed a reproducible growth lag when grown in LB (Figure 4.3, filled circles) presumably due to non-optimal ribosome function. This phenotype was not observed in either single mutant, indicating that *rpmGA* and *rpmGB* encode functionally redundant proteins and both are expressed under these conditions. Introduction of a *zur* mutation suppresses the poor growth phenotype of the *rpmGA rpmGB* double mutant, but only in the strain in which the frameshift mutation in *rpmGC* has been corrected (Figure 4.3, open triangles). Thus, *rpmGC* encodes a functional L33 protein. This also raises

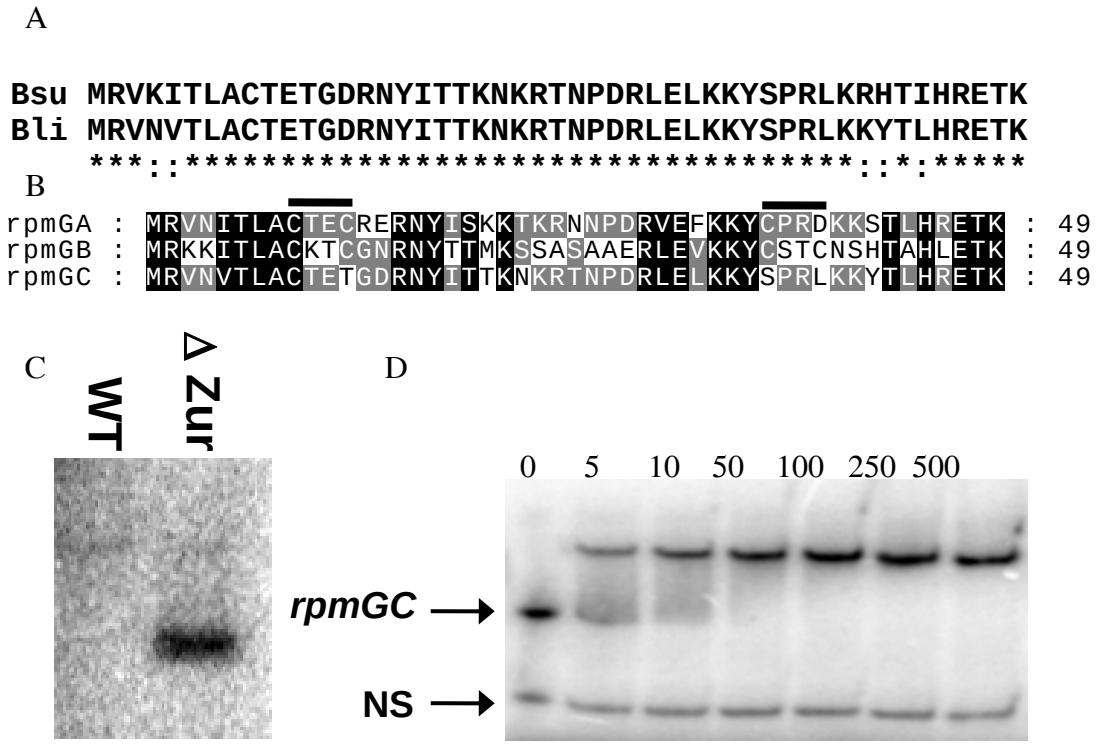



Figure 4.2 RpmGC characterization A. Protein sequence alignment of the L33 C-proteins of *Bacillus subtilis* (with the frameshift mutation corrected) and *Bacillus licheniformis* (Bli). B. Amino acid conservation of *B. subtilis* L33 C+ and C- paralogs. Black bars highlight the CXXC motif which binds a structurally important Zinc cofactor in C+ proteins. C. Northern Blot of RNA isolated from CU1065 (WT) and a *zur* mutant showing the *rpmGC* transcript is only produced when Zur is absent. D. Zur binds with high affinity to the *rpmGC* promoter in this electromobility shift assay using purified ZurBS protein, the *rpmGC* and a nonspecific (NS) promoter region with protein concentrations (nM) increasing left to right.

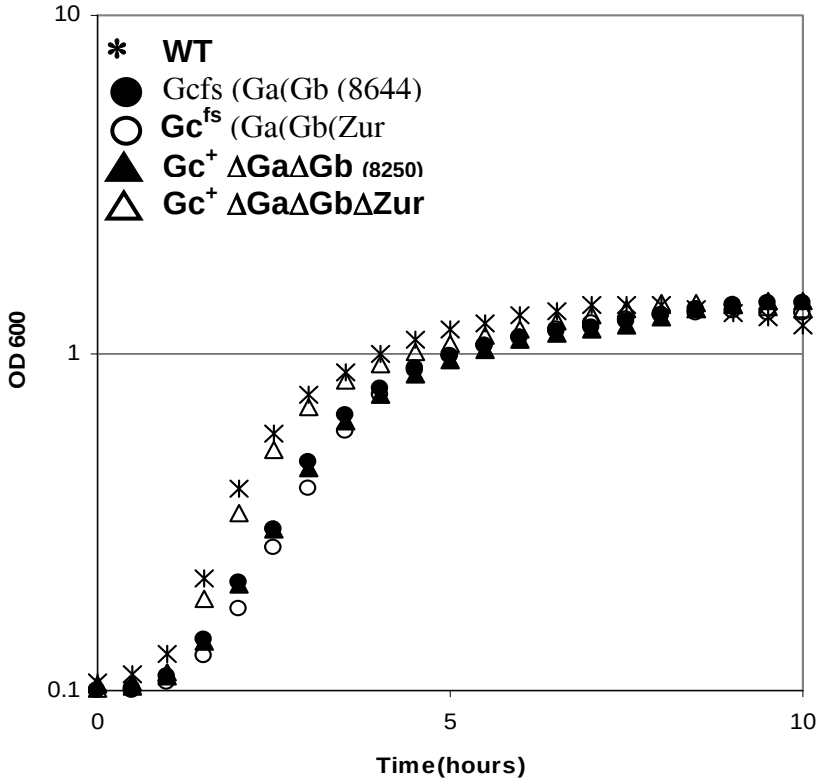



Figure 4.3 RpmGC growth curve. A *zur* mutation restores a wild-type growth rate to a strain lacking RpmGA and RpmGB only if the gene encoding RpmGC is corrected so as to encode a complete protein product (HB8251, open triangles). All other disruption combinations which do not restore the WT growth rate are shown as controls with their strain number listed in parentheses (see Table 4.1 for genotypes). The *rpmGC* frameshift mutation is denoted 'fs' and the corrected *rpmGC* '+'.

the possibility that L33c might function, as also postulated for the L31 paralog YtiA, in mobilization of zinc as part of the zinc starvation response.

**Zinc content of the ribosome.** Critical to the hypothesis that Zur-regulated r-proteins act to mobilize zinc from the ribosome is the presence, on the ribosome, of C+ proteins, which have bound zinc. While the previous characterization of RpmE and YtiA showed zinc occupancy of only RpmE, these experiments relied on protein expression in *E. coli* and use of a epitope tagged protein and column purification (14). Here, we sought to measure total ribosome-associated zinc. We reasoned that a ratio of at least three zinc atoms per ribosome would be expected due to the presence of three known C+ ribosomal proteins (S14, L31, L33). However, L32 (*rpmF*) and L36 (*rpmJ*) of *B. subtilis* also have CxxC motifs and it is possible that they also bind zinc.

Using a PAR-based assay, we monitored zinc release from ribosomes upon denaturation with boiling and SDS. Our wild-type strain was found to have ~2.5 Zn/ribosome (Table 4.3), in close agreement with our expectation and in the same range as similar measurement of the zinc content of yeast ribosomes (3). This should be considered as a lower limit for the Zn/ribosome ratio since loosely associated r-proteins (including, for example, L31) are easily lost during purification (4) and, conversely, even these harsh conditions may not fully denature the ribosome and release all of the bound zinc. As expected, the Zn/ribosome ratio was reduced in strains missing one or more of the C+ r-proteins. Loss of L31 (and possibly L33) from the ribosome during purification may explain why ribosomes from strains missing the C+ L31 or L33 proteins each display a decrease of only ~.5 Zn/ribosome as compared to WT (Table 4.3). Alternatively, these proteins may be stoichiometrically associated with the ribosomes but not fully saturated with zinc. It is difficult to quantify the stoichiometry of r-proteins in purified ribosome preparations with sufficient precision to distinguish between these two hypotheses. Nevertheless, we do consistently

Table 4.3 Zinc content of ribosomes purified from cells containing disruptions of genes encoding nonessential C+ ribosomal proteins.

| | **Zn per ribosome (S.D)[1]** |
|---|---|
| Wild Type (CU1065) | 2.5(.15) |
| *rpmE* (HB8608) | 2.0(.40) |
| *rpmGA, rpmGB* (HB8644) | 1.9(.24) |
| *rpmE,rpmGA,rpmGB* (HB6916) | 1.5(.33) |

[1]Values are averages of zinc content as determined by PAR assay normalized to ribosome number from at least 3 independent purifications with standard deviations in parentheses.

observe a decrease in ribosome-associated zinc in cells lacking one or more zinc-containing (C+) r-protein.

Since ribosomes are the most abundant macromolecular complex in the cell (with >50,000 copies in a rapidly growing cell), even 2 or 3 zinc atoms per ribosome represents an enormous reservoir of zinc (>$10^5$ zinc atoms). Previous estimates place the total zinc content of logarithmically growing *E. coli* cells in the vicinity of 2 x $10^5$ atoms per cell and this value seems to be a relatively constant function of cell size (16). Thus, ribosomes may easily account for the majority of the zinc in the cell. Mobilization of this stored zinc might provide a significant growth advantage under zinc-limiting conditions as it would enable the cell to continue r-protein, and consequently ribosome, synthesis.

**Creation of zinc starvation conditions**. We next aimed to test whether the induction of Zur controlled r-proteins can 'mobilize' stored zinc from the ribosome by monitoring their effect on growth under conditions of severe zinc limitation. Because bacteria have extremely effective zinc uptake systems, they are able to grow well in media to which no zinc has been added, presumably due to trace contamination of other reagents with zinc. Indeed, most studies of zinc starvation have used strong metal ion chelators to impose zinc limitation. Unfortunately, this approach introduces additional complexities since chelators often impose limitations for multiple required metal ions. In *E. coli*, extraordinary efforts have been required to generate reproducible zinc-limiting conditions (<60 nM zinc) in chemostats (8). In preliminary studies, we also found that *B. subtilis* grew to high cell densities even upon repeated sub-culturing in a defined minimal medium containing no added zinc, even when all reagents were of the highest available purity and were Chelex-treated to reduce trace metal contamination.

To more effectively create zinc-limiting growth conditions, and to increase the cell's dependence on the hypothesized ability to mobilize zinc stored internally, we genetically inactivated high affinity zinc uptake. First, we deleted the high affinity zinc transporter controlled by Zur, *ycdHIyceA.* The *ycdHIyceA* mutant strain displayed a decrease in both growth rate and yield as compared to WT in our zinc-starvation minimal medium (ZSMM). In this genetic background, the additional disruption of *citM*, a transporter of metal-citrate complexes, or *zinT*, a candidate zinc chaperone (8), did not further decrease growth (filled shapes Figure 4.4). In contrast, when *yciC*, a proposed metallochaperone, was also deleted a more severe growth defect was observed (open shapes Figure 4.4). These growth deficiencies are seen in ZSMM, but not during growth in rich media such as LB, consistent with the notion that they are due to zinc starvation. Moreover, growth can be completely restored by addition of zinc (Figure 4.4B), but not other metals. For further studies, we have focused on the roles of r-proteins in the double mutant background lacking high affinity zinc uptake (a *ycdH* mutation) and the *yciC* metallochaperone which we hereafter refer to as the HC mutant strain.

**The L31 ribosome protein contributes a physiological relevant source of zinc.** To begin to characterize the contribution of each of the Zur-controlled r-proteins in growth under zinc-limiting conditions, we disrupted *ytiA* and y*hzA* individually and together in the HC mutant background (Figure 4.5). Deletion of either or both *ytiA* and *yhzA* clearly exacerbate the growth defect in ZSMM (Figure 4.5). It should be noted that in this background *rpmGC* contains a frameshift mutation. However, similar results were seen in strains in which this frameshift mutation was corrected (data not shown and see below).

The growth defect due to the disruption of *ytiA* (encoding the C- L31 paralog) may result from either or both of two scenarios. First, it is possible that the lack of

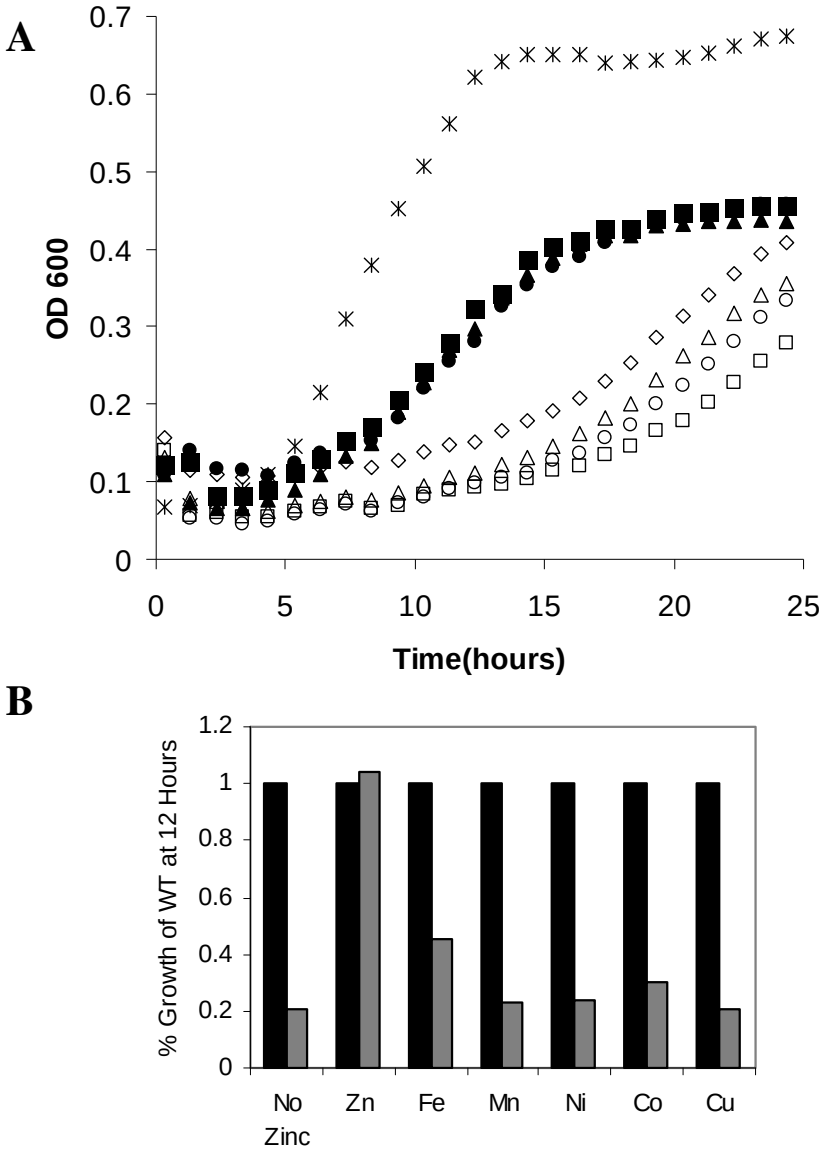



Figure 4.4 Mutation in zinc uptake and homeostasis proteins leads to growth defects in zinc starvation minimal medium. A. WT (asterisks) cells grow well in this medium. Cells defective for the *ycdH* encoded ABC transporter grow more poorly (filled shapes) but there is no additional defect due to deletion of *citM* (filled circles-6865), *zinT* (filled triangles-6867) or both *citM* and *zinT* (filled squares-6869). In contrast, a *ycdH yciC* double mutant is significantly growth impaired (open diamonds-6866), and with the additional mutation of *citM* (open circles-6868), *zinT* (open triangles-6890) or both *citM* and *zinT* (open squares-6871) the growth defect phenotype now increases. B. Only zinc is able to fully restore the growth defect of the *ycdH, yciC* double mutant observed in A. Growth at 12 hours for WT (black bars) and the *ycdH, yciC* double mutant strain (grey bars) plus 1μM added metals as labeled. Values are shown as a percent of WT growth in each condition.

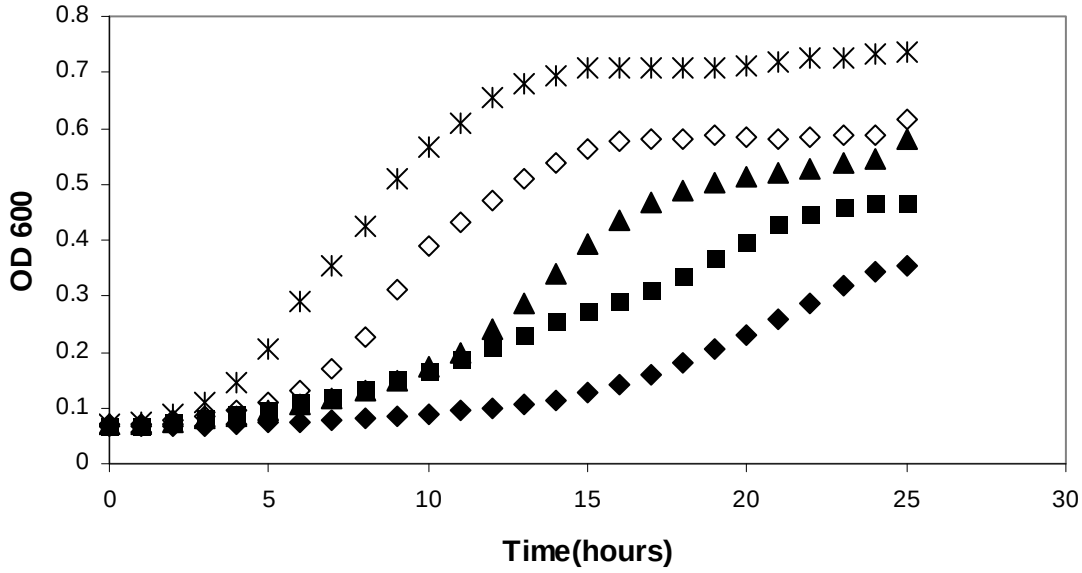



Figure 4.5 Zur-regulated r-protein paralogs contribute to growth under severe zinc starvation conditions (in the HC double mutant background growing in ZSMM). While WT (asteriks) grows well, *ycdH yciC* (open diamonds-6866) shows a reproducible lag and decrease in yield. This growth defect is magnified in the *ycdH yciC ytiA* (closed traingles-6882), *ycdH yciC yhzA* (closed squares-6880), and *ycdH yciC ytiA yhzA* (closed diamonds-6883), mutant strains.

available zinc prevents the C+ L31 (*rpmE* gene product) from folding properly and the lack of the Zur-regulated paralog leads to a situation in which ribosomes no longer have a functional L31 protein. As noted previously (1), and confirmed here (data not shown), an *rpmE ytiA* double mutant does have a modest growth defect in rich medium, consistent with the hypothesis that L31, while not an essential protein, contributes to ribosome function even under non-zinc limiting conditions. Second, it is possible that the growth defect observed in the ZSMM is due to an inability of the cells to mobilize zinc stored in the ribosome (in the L31 C+ protein) by displacement by newly synthesized C- L31 (YtiA). It has previously been shown that addition of purified YtiA to ribosomes displaces L31 (14).

To distinguish between these two scenarios we created a strain in which both L31 genes (*ytiA* and *rpmE*) were deleted in an HC double mutant background. We then placed each gene back into this strain, at an ectopic locus, under the control of the constitutive *rpmE* promoter thus removing the Zur control over *ytiA*. We reasoned that in these strains the cells would contain ribosomes complete with one or the other

L31 protein. Upon shifting to ZSMM, the induction of YtiA would displace the C+ L31 (in the HC $P_{rpmE}$-*rpmE* strain) and thereby mobilize zinc. In contrast, if the pre-existing ribosomes were assembled with the C- L31 (in the HC $P_{rpmE}$-*ytiA* strain) there would be no advantage gained by the derepression of the Zur-regulated *ytiA* gene. Neither would there be a defect in the ribosomes due to a lack of L31 since they are provided with a constitutively expressed and functional L31 encoded by the $P_{rpmE}$-*ytiA* gene. Indeed, the strain constitutively expressing only *ytiA* (Figure 4.6 open circles) grew more like the *ytiA* mutant (Figure 4.6 closed triangles) than the HC double mutant (Figure 4.6 closed squares). These results provide strong support for the inference that Zur-regulated expression of YtiA mobilizes zinc from the ribosome by displacement of L31. Whether zinc is spontaneously released from the small L31

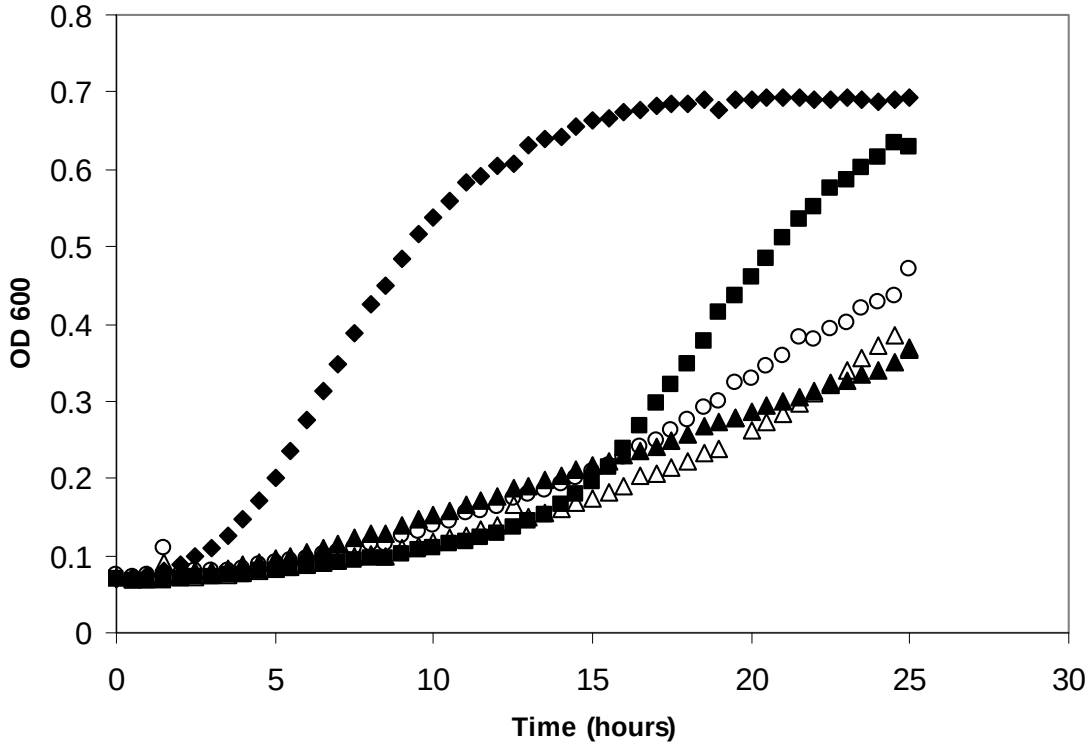



Figure 4.6 L31 deletion growth defect is primarily due to the inability to mobilize stored zinc from the ribosome. WT (closed diamonds) grows well in ZSMM and shown for reference. The strain which has only the L31 C- ribosomal protein YtiA being expressed (open ciricles-6984) has a similar growth rate and lag as *ycdH yciC ytiA* (closed triangles-6882) and not *ycdH yciC* (closed squares-6866) suggesting zinc mobilization is responsible for the observed growth defect. Strain expressing only the L31 C+ protein RpmE (open triangles-6983) is shown as a control.

peptide (66 amino acids) or whether this requires an unfoldase or proteolysis is presently unknown.

**Production of a C- S14 paralog is important for growth under zinc limitation**. It has been previously proposed that the Zur-regulation of the S14 paralog, YhzA, allows continued ribosome synthesis even when cells are deprived of zinc. Since S14 is an essential protein ((15), and data now shown), this is difficult to verify genetically. However, this model predicts that *yhzA* mutants should be growth impaired upon transfer to ZSMM since newly synthesized S14 (RpsN) will be deprived of its needed zinc cofactor and the cells must therefore rely on previously assembled ribosomes for growth. Indeed, the HC *yhzA* mutant cells grew much more poorly in ZSMM than the HC parent strain. It is also interesting to note that growth of this strain appears to be linear with time, rather than exponential, consistent with an inability of the cells to increase the total ribosome number. Linear growth is postulated to reflect the balance between increasing cell numbers and a decreasing growth rate as ribosomes become limiting for growth, although further studies will be required to test this model.

**RpmGC does not contribute significantly to the zinc starvation response**. It is clear that both YtiA and YhzA contribute to the cell's ability to adapt to zinc deprivation as monitored by growth of the transport-defective HC strain in ZSMM. Next, we sought to determine if L33c (encoded by rpmGC) provides a significant growth advantage and, further, whether it might function to mobilize zinc from the constitutively expressed C+ L33 proteins. The same corrected rpmGC construct shown previously to complement an L33 disrupted strain (Fig 4.3), was integrated into the HC double mutant, the single *ytiA, yhzA* and the double *ytiA yhzA* mutant strains (all in an HC mutant background). We hypothesized that if any of the growth phenotypes we had observed previously with *ytiA* or *yhzA* disruptions were due, in whole or in part, to the lack of L33c, introduction of a functional *rpmGC* gene would

increase fitness. However, in all cases strains containing the corrected *rpmGC* gene (Supp. Figure 4.1-filled symbols) showed no significant growth improvement when compared with strains containing the original frameshift mutation. Thus, L33c provides no significant advantage to the cell under these growth conditions, even though it can complement the *rpmGA rpmGB* double mutant for growth in rich medium (Fig. 4.3). This suggests that either L33 proteins do not provide a mobilizable pool of zinc or that, under these conditions, the L33a and L33b proteins are still able to obtain sufficient zinc for function.

**5. Concluding Remarks**

*B. subtilis* encodes at least three sets of paralogous r-proteins: S14 (*rpsN* and *yhzA*), L31 (*rpmE* and *ytiA*) and L33 (*rpmGA, rpmGB* and *rpmGC*). Previous work led to the proposal of two distinct Zur-mediated mechanisms for responding to zinc limitation (1, 14). The Zur-regulated L31 paralog, YtiA, was shown to actively displace the loosely associated C+ protein, RpmE, from the surface of the ribosome (1). It was speculated that YtiA-mediated release of L31 from the ribosome, followed perhaps by proteolysis, would mobilize zinc for use as a cofactor for essential proteins. It is possible that the paralogous L33 proteins have a similar function. In contrast, the essential zinc-requiring S14 protein (*rpsN*) is buried deep within the ribosome structure and is required for assembly. Therefore, induction of the Zur-regulated C-S14 paralog (YhzA) was postulated to provide a 'fail-safe' mechanism for continued ribosome assembly under zinc limiting conditions (15). While these are reasonable models, the challenges of limiting cells for zinc have precluded detailed physiological tests of these ideas.

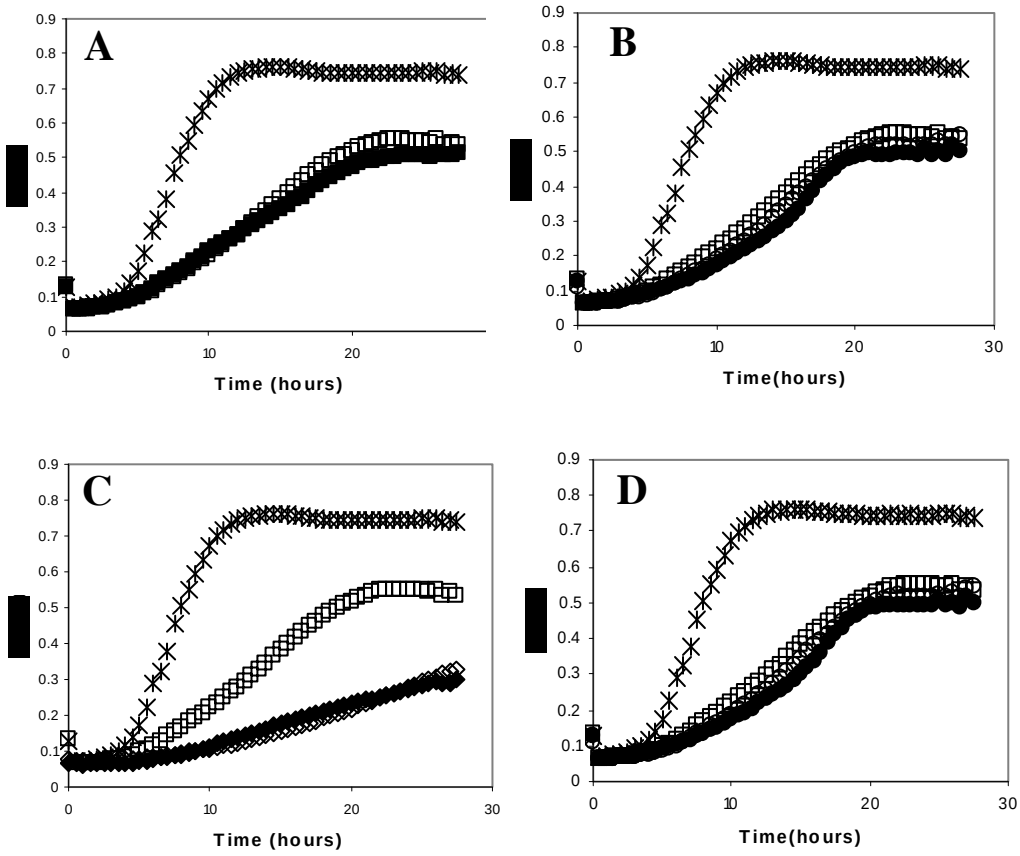
Here, we have explored the functions of the Zur-regulated C- paralogs in adaptation to conditions of severe zinc deprivation. Since *B. subtilis*, like many

bacteria, has extremely efficient zinc scavenging mechanisms it is difficult to deprive cells of zinc by simple omission from the growth medium. We have increased zinc stress by genetic ablation of both high affinity zinc uptake (the *ycdHIyceA* operon) and the YciC metallochaperone. Using this HC strain, we demonstrate that both the Zur-regulated L31 (YtiA) and S14 (YhzA) paralogs contribute to adaptation to zinc deprivation. In the case of L31, our growth studies indicate that this is due to an inability of YtiA to mobilize zinc. Conversely, the growth properties of the *yhzA* mutant strain are consistent with, and provide support for, the previously proposed "fail-safe" mechanism of continued ribosome assembly under zinc limitation. Finally, we have provided evidence that the previously noted *rpmGC* gene can encode (once the frameshift is corrected) a functional L33 protein but we find no evidence, under our growth conditions, to suggest that this protein provides an advantage for growth under zinc limiting conditions.

In general, the ribosome is a highly conserved structure and most r-proteins are encoded by essential genes. However, some r-proteins are not highly conserved, appear to be dispensable for growth, and are variably associated with ribosomes during purification. Whether these are *bona fide* r-proteins, or merely proteins with other functions that associate with the ribosome, is not always clear. The finding that L31 functions in storage and mobilization of zinc is one such example. Since cells lacking any L31 are reduced in growth even in rich medium, its seems likely that this protein does play some role in ribosome function. However, it also appears to play a role in storing and mobilizing zinc, in which case L31 can be considered as a dual function protein.

The extent to which ribosomal proteins may have extra-ribosomal functions has recently become better appreciated (19). From bacteria to humans, r-proteins are involved in a variety of functions including roles as a DNA endonucleases (20),

regulator of macrophage migration inhibitory factor (5), modulator of RNase E activity (18), and a  factor in DNA regulation (10) just to name a few. The finding of paralogous pairs of r-proteins with one partner regulated by Zur (or other zinc-sensing transcription factors) suggests that zinc mobilization might represent another extra-ribosomal function for r-proteins. The physiological studies reported here, specifically for L31, provide evidence that this mechanism is indeed operative. Since zinc-regulated, C- r-proteins are widespread in the bacteria, it is likely that this represents a widespread adaptation to conditions of zinc limitation.




Supplemental Figure 4.1 *rpmGC* has no affect on strain's ability to grow in zinc starved conditions. All strains have *ycdH* and *yciC* deleted as a starting point with the exception of WT (asterisks). Additionally all filled symbols have rpmGC+ while open symbols have rpmGCfs A. *ycdH, yciC (*squares) B. *ytiA (*circles*)* C. *yhzA* (triangles) D. *ytiA yhzA* (diamonds)

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# APPENDIX

## CELL WALL STRUCTURES AND ANTIBIOTIC ACTIONS*

### 1. Description

A small group activity which uses a problem based approach to introduce students to the differential action of antibiotics on the components of bacterial and archaeal cell walls.

### 2. Summary

This problem-based exercise challenges students to think critically about the connection of antibiotics to cell-wall structure. Without prior knowledge of antibiotics' mode of action, students are asked to deduce how the differential components of bacterial cell walls create an observed antimicrobial activity on a panel of microorganisms. Our format employs three distinct approaches to frame student understanding: first, small groups of 10-15 students provide a space for active discussion and peer-teaching; second, students apply their knowledge to solve an activity based on an experimental scenario; finally, the process of lecture, discussion and small groups is iterative.

* Scott E. Gabriel, Letal Salzberg, Bronwyn Butcher, Sue Merkel ASM Microbe Library 2007

**3. Learning objectives**

At the completion of this activity students will be able…

- To describe the function of various cell wall structures of bacteria and archaea.
- To relate cell wall structure to the effectiveness of antimicrobial compounds.
- To explain that cell membranes are common to all bacteria and archaea and can be a target for antimicrobial compounds.

**Suggestions for determining student learning**: The primary mechanism for determining student learning is the answers they provide to the questions on the worksheet. We have also used as a secondary assessment tool a pre and post test given to students the first and last day of lab recitation (see field testing section for details).

**Field Testing** During the Spring of 2007 we developed a pre/post test (see attached document) that was given to students at the first lab recitation session before any small group or lab activity covered the material being tested. This same test was also given to students on the last day of lab recitation. With this design, our data provides insight into student learning and the level of material retention. Using questions 1,4 and 5 from the test we quantified student learning on the subject of function of cell wall and antibiotics. Test scores for question #1 improved from 68% (n=75) to 90% (n=71), scores on question #4 improved from 68% (n=75) to 90% (n=71) where scores for question #5 improved from 61% (n=75) to 92% (n=71).

**4. Small Group Activity: Cell Wall Structures and Antibiotic Action**

You have recently been hired as an Assistant Professor where your research deals with isolating novel antibiotics to aid in the growing problem of antibiotic resistance. In your first months of work, you have isolated several interesting antimicrobial compounds which show real promise. Unfortunately, late one night you did not label the tubes clearly. Now your promising work needs to be pieced back together with only a few scraps of information scribbled on a napkin from that night.

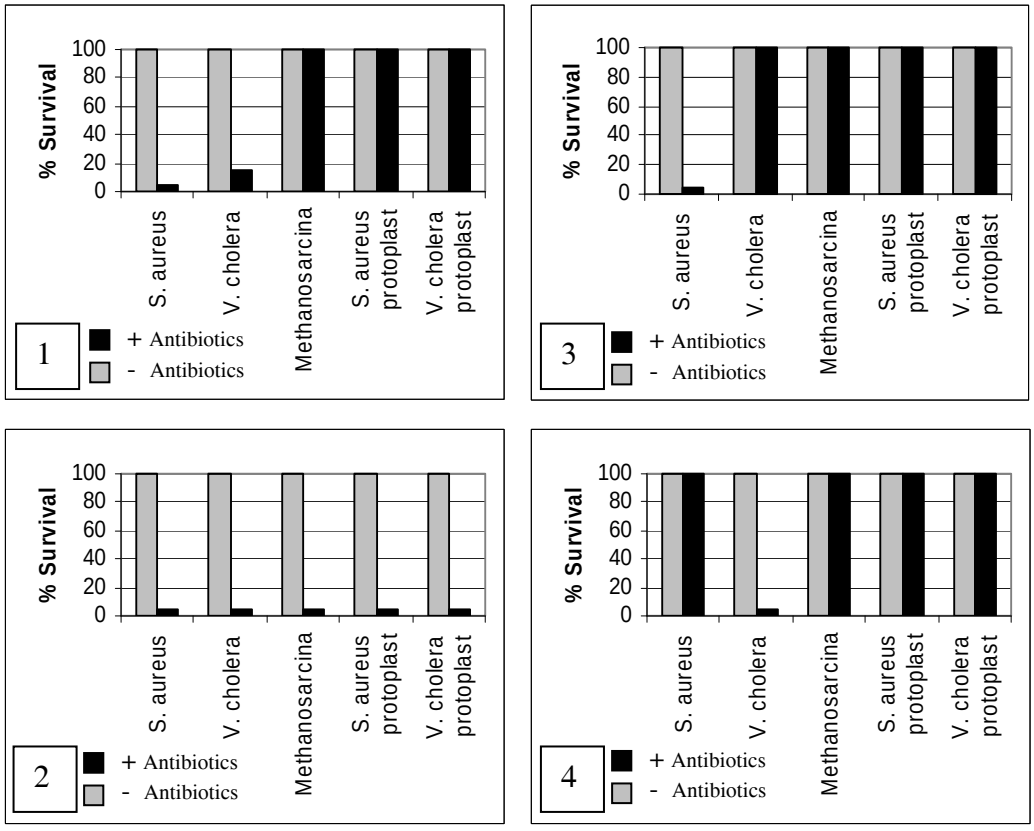
**Deciphered napkin scribblings….**

Antibiotic A: 0.5 kDa protein, targets peptidoglycan
*983-7554 cutie from Castaways*
Antibiotic B: 20 kDa protein, targets peptidoglycan
Antibiotic C: Cationic antimicrobial peptide
*get!! milk, bread, cheese, stamps*
Antibiotic D: Targets LPS *Bus#81 comes at 7:10*

With some more searching, you found these results in your notebook. Unfortunately they are not labeled either. These graphs (see Fig Appendix 1) represent **percentage survival of bacteria or protoplasts after treatment with the antibiotics.** Controls with no antibiotic added (Grey Bars); Samples with antibiotic added (Black bars)

**Helpful background information:**
*Staphylococcus aureus* : Gram-positive Bacterium
*Vibrio cholera*: Gram-negative Bacterium
*Methanosarcina*: an Archean Bacterium

**Cationic Antimicrobial Peptides (CAMPs):** These positively charged antibiotics are attracted to the negatively charged cell wall and membrane. They are hydrophobic, and they insert into the membranes to create pores.




Appendix Figure 1 Novel antibiotic candidate data. Recovered data for small group exercise. Graphs 1-4 each correspond directly to one of the four antibiotics (A-D)

**Small Group Activity — Cell Wall Structures and Antibiotic Action**
**Writing Assignment**

**Your Name:**
**Your Instructor's name:**

*Complete the following questions LIMITING your answers to the spaces provided*

1. **Using the information on the previous page, match each antibiotic with an experiment. Fill in the blanks below and briefly discuss the results observed for each experiment, making sure to include the predicted mode of action for each antibiotic. (9 points)**

Experiment #1 is Antibiotic _____

Experiment #2 is Anitbiotic _____

Experiment #3 is Antibiotic _____

Experiment #4 is Antibiotic _____

2. **Pick one of the antibiotics above (A-D) and describe how a bacterial cell <u>could become resistant</u> to that antibiotic. In other words, what in the cell would have to change to make a bacterium resistant? (1 point)**